UNIVERSITY OF ALBERTA

FACTORS AFFECTING THE DISPOSITION OF VERAPAMIL ENANTIOMERS IN HUMAN AND RAT

BY

MUHAMMAD MASOOD BHATTI

©

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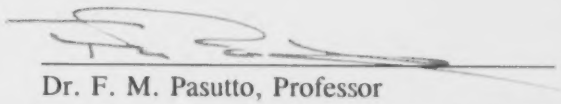

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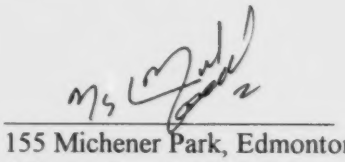

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DEDICATIONS

To my parents Muhammad Ahmed Bhatti, Hussan Ara Bhatti, my wife Tayyiba Bhatti and children Nizamodean and Zainulabedean.

ABSTRACT

Verapamil (V) is a chiral calcium channel blocking drug used in the treatment of angina pectoris, hypertension and supraventricular tachycardia. Although V is manufactured and administered as the racemate, most of the calcium channel blocking activity resides with the S-enantiomer. V is extensively metabolized by the liver with a high extraction ratio. Therefore, the processes or drugs that alter hepatic blood flow and/or enzyme activity have potential to change the disposition of V enantiomers. Furthermore, the presystemic metabolism of V is nonlinear, and the stereoselective kinetics of racemic drugs, with high hepatic extraction ratios and nonlinear presystemic metabolism, are very susceptible to input-rate changes. Therefore stereoselectivity in the nonlinear metabolism of V may be affected by the rate of drug entry into the portal vein after its oral administration. The objectives of this project were to study the influence of input-rate and co-administration of metoprolol (a β-blocker) on the stereoselective pharmacokinetics of V enantiomers.

Two stereospecific HPLC assays were developed, one involving chiral derivatization (in a microwave) with (-)-menthyl chloroformate and the other using a chiral stationary phase. These HPLC methods can be used to evaluate the pharmacokinetics of V and norverapamil (NV) enantiomers in human and rat plasma.

In healthy volunteers, significantly higher plasma concentrations of R-enantiomers of V and NV were found after the administration of immediate-release (IR) and controlled-release (CR) formulations of racemic V. The oral clearance of V was stereoselective in favor of the S-enantiomer regardless of formulations. There

was a general trend for more active S-V to be decreased after CR administration, when compared with IR administration. This reduction may be related to slower drug input rate from the CR formulation.

The pharmacokinetics of V was studied in rats after intravenous and oral administration of racemic V. As in humans, V was stereoselectively metabolized by rat and the oral bioavailabilities of both enantiomers were low. The stereoselectivity in the systemic and presystemic clearance was in the opposite direction in rat than in human. The protein binding of V and NV was stereoselective in rat (in favor of S-enantiomer) and humans (in favor of R-enantiomer). Age and NV did not have any affect on the binding of V enantiomers in both species.

Co-administration of metoprolol with V resulted in a significant increase in the oral bioavailability and significant decrease in the systemic and oral clearance of V enantiomers. The observed decreased systemic clearance of V enantiomers may be due to metoprolol induced decreased hepatic blood flow. Whereas, the increased oral bioavailability may be related to inhibition of hepatic isozymes. The latter possibility was investigated by examining the loss of V in the rat liver microsomal preparation, with and without metoprolol. The loss of V enantiomers was significantly slower in the presence of metoprolol. The study indicated that metoprolol altered both the intravenous and oral kinetics of V by apparently two different mechanisms; i.e. decrease in hepatic blood flow and inhibition of presystemic metabolism. Both the effects of metoprolol were non-stereoselective.

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GLOSSARY OF ABBREVIATIONS

α	selectivity of chromatographic peaks
AAG	α_1-acid glycoprotein
ANOVA	analysis of variance
AUC	area under the plasma concentration-time curve
AV	atrioventricular
β	elimination phase rate constant
C_{last}	last concentration point
CHF	congestive heart failure
Cl/F	oral clearance
Cl_s	systemic clearance
cm	centimeter(s)
CV	coefficient of variation
° C	degrees Celsius
D	dose
ECG	electrocardiogram
EOA	ethanolamine
f_u	free-fraction in serum
g	gram(s)
g	acceleration due to gravity

h	hour(s)
HPLC	high-performance liquid chromatography
"	inches
i.d.	inside diameter
inf.	infinity
I.S.	internal standard
ISA	intrinsic sympathomimetic activity
i.v.	intravenous
kg	kilogram(s)
λ_n	terminal elimination rate constant
l	liter(s)
M	male
MCF	(-) menthyl chloroformate
MTBE	methyl *t*-butyl ether
M	molar
µg	microgram(s)
µl	microliter(s)
mg	milligram(s)
min	minute(s)
ml	milliliter(s)
n	number of observations

ng	nanogram(s)
nm	nanometer
NV	norverapamil
o.d.	outside diameter
R_s	chromatographic resolution
r^2	squared correlation coefficient
r	correlation coefficient
s	second(s)
SD	standard deviation
SEM	standard error of the mean
τ	dosing interval
TEA	triethylamine
tid	three times daily
$t_{1/2}$	half-life
U	unit(s)
UV	ultraviolet
V	verapamil
Vd_β	apparent volume of distribution

1. INTRODUCTION

Calcium and Calcium Channels

Calcium is involved in several major cellular processes, including contraction, secretion and neural activity. As a free cation or after the formation of a complex with some macromolecules, calcium is an activator of several enzymes of the cell. The primary role of calcium to control cellular activity was first recognized by S. Ringer in 1883, who reported the necessity of a small amount of calcium in the physiological solution to allow maintenance of cardiac contractility *in vitro*. Calcium is not only the regulator of metabolic pathways and transforming external messages into appropriate cellular responses, but, when in excess in the cellular medium, it may produce overactivation of several processes, leading to cell death.

To control the vast difference in the concentration between calcium in the extracellular fluid and the cytosol, requires regulation of the calcium transmission across the sarcolemma. There are several possible modes of calcium entry. Calcium can enter *via* the calcium channels, *via* sodium/calcium exchange mechanism and it can 'leak in' along the concentration gradient. Of all these possibilities, the calcium channels are thought to provide the chief mechanism for calcium ion entry [1].

Calcium channels may be identified by combined electrophysiological and pharmacological techniques. Tsien and colleagues [1-2] identified different channels, which they called L, T and N, according to their sensitivity to membrane potential variation and to the time required to reach inactivation.

The long-lasting or L-type channels are also called high-voltage activated channels. They are the predominant type of voltage-dependent calcium channels in the heart and smooth muscles. They are the main pathway for calcium entry, which support the contraction of depolarized arteries, or for the calcium-induced calcium-release mechanism of cardiac muscle. They are slow inactivating and fast deactivating.

The transient or T-type channels are also called low-voltage activated calcium channels because they are opened by small depolarizations from negative holding potentials. They exhibit fast inactivation and slow deactivation. T-type channels are found in a wide variety of excitable and non-excitable cells, but are absent in adrenal chromatin cells and sympathetic neurons. It is believed that the function of T-type channels is to support pacemaker activity.

The nervous or N-type calcium channels are also high-voltage activated channels. The main difference from L-type is pharmacological. N-type channels are resistant to dihydropyridines, but blocked by ω-conotoxin. It is proposed that N-type channels are located on nerve terminals [3].

Calcium Channel Blockers

In 1964, Fleckenstein reported that the chemical agents, verapamil and prenylamine, had the same inhibitory effect on cardiac muscle as did the withdrawal of extracellular calcium ions [4]. Since that time, a large number of organic compounds of widely different chemical structures, have been found to have similar inhibitory effects [5-6]. These effects are primarily due to the direct action of these compounds on calcium channels. The members of this group of compounds were originally called calcium antagonists because their inhibitory effects could be reversed by increasing the calcium ion concentration in the extracellular medium. More recently, they have also been referred to as calcium entry blocking agents or calcium channel blockers. Calcium channel blockers are chemically heterogeneous and include analogs of benzothiazepine, phenylalkylamine and dihydropyridine. The L-type calcium channels which are responsible for calcium entry during the plateau of the action potential are sensitive to diltiazem, nifedipine and verapamil, the three prototype calcium channel blockers. These three agents, with different molecular structures, bind at allosterically-linked set of sites on a major protein of the L-type of vascular and myocardial calcium channels. These sites represent the active part of the calcium channel and interaction at these binding sites block channel functions [7].

[1]VERAPAMIL

Verapamil is the prototype of the phenylalkylamine class of calcium channel blockers. Initial studies with verapamil showed that it exerted a negative inotropic effect on isolated cat and rabbit myocardium in addition to its vasodilator properties [8]. These potent negative inotropic effects seemed to distinguish this drug from the classical coronary vasodilators such as nitroglycerin and papaverine, which are potent vasodilators but have little, if any, myocardial depressant effect. Fleckenstein and coworkers [9] were among the first to differentiate between the effects of β-adrenergic antagonists and this new compound.

The next advance in the understanding of this agent was made by Singh and Vaughan-Williams [10]. They had previously proposed a classification of antiarrhythmic compounds [11], separating them into local anesthetics, β-blockers and a third class which prolonged the duration of the cardiac action potential. However, none of these effects explained the antiarrhythmic effect of verapamil. In 1972, they proposed a fourth class of antiarrhythmic drugs [10], typified by verapamil, and they clearly separated its effects from those of sodium channel inhibitors and β-blockers. They proposed that verapamil exerts its antiarrhythmic actions and its negative inotropic effects through interference with calcium conductance. This idea has now been expanded and our understanding of the

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[1] Verapamil is referred to racemic verapamil throughout the thesis, unless specified otherwise.

importance of calcium ions in excitation-contraction coupling has greatly increased [6].

Physicochemical Properties of Verapamil

The chemical structure of verapamil, α-[3-[[2-(3,4-dimethoxyphenyl)ethyl]-methyl amino]propyl]-3,4-dimethoxy-α-(1-methylethyl)benzeneacetonitrile; is depicted in Figure 1-1. As the hydrochloride salt, it is an odorless, white crystalline solid [12]. The molecular weights of the base and hydrochloride salt are 454.61 and 491.07, respectively. Verapamil is a relatively lipophilic calcium channel blocker, which is reflected by a water/*n*-octanol partition coefficient (log P value) of 3.79 [13].

Verapamil base is practically insoluble in water. It is sparingly soluble in hexane, soluble in benzene, ether, lower alcohols, acetone, ethyl acetate and chloroform [12]. Verapamil hydrochloride is sparingly soluble in water. It is soluble in ethanol, isopropranol, acetone, ethyl acetate and methanol [12]. The pka value for verapamil has been reported to be 8.9 [13].

Utilizing a Uni-Melt capillary melting point apparatus (Arthur H. Thomas Company, Philadelphia, PA), the melting point of racemic verapamil hydrochloride was determined to be 140.8° C. The melting point of racemic verapamil hydrochloride has previously been reported to be within the range 138.5-140.5° C [12].

Pharmacology and Mechanisms of Action of Verapamil

Verapamil is a chiral calcium channel blocking drug which is manufactured and administered as the racemate. R-verapamil possesses less than 1/10 of the calcium channel blocking activity of its antipode [14].

The principal physiological action of verapamil is to inhibit the transmembrane influx of extracellular calcium ions across the membrane of myocardial cells and vascular smooth muscle cells. Calcium plays an important role in the excitation-contraction coupling process of the heart and vascular smooth muscle cells and in the electrical discharge of the specialized conduction cells of the heart. The membranes of the cells contain numerous channels that carry a slow inward current and are selective for calcium. Activation of these slow calcium channels contributes to the plateau phase of the action potential of cardiac and vascular smooth muscle cells. Verapamil is thought to inhibit ion-control gating mechanisms of the channel, deform the slow channel, and/or interfere with the release of calcium from the sarcoplasmic reticulum [15]. By inhibiting calcium influx, verapamil inhibits the contractile processes of cardiac and vascular smooth muscle, thereby dilating the main coronary and systemic arteries. In patients with vasospastic angina, inhibition of spontaneous and ergonovine-induced coronary artery spasm by verapamil results in increased myocardial oxygen delivery. Dilation of systemic arteries by verapamil results in a decrease in total peripheral resistance, systemic blood pressure, and the afterload of the heart. Decreases in peripheral vascular resistance usually occur without orthostatic decrease in blood

pressure or reflex tachycardia. The reduction in afterload, seen at rest and with exercise, and its resultant decrease in oxygen consumption are thought to be responsible for the effects of verapamil in patients with unstable and chronic angina pectoris [16-18].

In contrast to nifedipine, verapamil has substantial inhibitory effects on the cardiac conduction system and is considered as a class IV antiarrhythmic agent [19]. Although verapamil rarely produces clinically important changes in the rate of sinoatrial (SA) node discharge or recovery time, it may reduce the resting heart rate and produce sinus arrest or SA block in patients with a SA node disease (e.g., sick sinus syndrome). Verapamil also slows conduction and prolongs refractoriness in the atrioventricular (AV) node, thereby prolonging the atria-His bundle (AH) interval. This usually also results in PR-interval prolongation on the ECG, which is correlated with plasma verapamil concentration [20-24]. In patients with paroxysmal superventricular tachycardia, verapamil's effects at the AV node result in an interruption of the reentrant pathway and restoration of normal sinus rhythm. Similarly, its effects on the AV node reduce the rapid ventricular rate caused by atrial flutter and/or fibrillation. Verapamil may depress velocity of depolarization and amplitude and prolong intra-atrial conduction times in diseased or depressed but not normal atrial tissue [24]. Verapamil does not alter normal AV conduction, but acceleration of ventricular rate and/or ventricular fibrillation can occur in patients with atrial flutter or fibrillation [25-27].

Adverse Effects of Verapamil

Adverse reactions to verapamil are usually mild, transient, require little or no therapy, respond promptly to drug withdrawal, and often represent an extension of known therapeutic pharmacological effects [24]. The majority of these side effects occur during dose-titration; most disappear spontaneously or after minor adjustment of dosage. Less than 6% of patients required either dose reduction or discontinuation of verapamil. Constipation, dizziness, hypotension, headache, peripheral edema and nausea are the most frequently reported adverse experiences with verapamil. Mild laxative, stool softeners, mild analgesics and diuretics have been used successfully in their management, where appropriate. Less than 2% of patients receiving verapamil experienced bradycardia, advanced AV block and congestive heart failure. Delayed adverse effects may occur following ingestion of the control-released preparations.

PHARMACOKINETICS OF VERAPAMIL

Absorption

Verapamil is rapidly and completely absorbed after oral administration [28]. Due to extensive first-pass hepatic metabolism, only 12-20% of the drug reaches the systemic circulation [29]. As with other drugs which are extensively metabolized by the liver, verapamil plasma concentrations show considerable

interindividual variations. Patients with liver disease have a significant increase in verapamil bioavailability [30]. Average time to peak plasma concentrations is between 1-2 hours after ingestion of the drug. After oral administration of 120 mg dose of verapamil every six hours, plasma levels ranged from 125-400 ng ml^{-1} [24]. Higher values have been reported occasionally [24]. The presence of food in the gastrointestinal tract seems to have no effect on the bioavailability of verapamil, but can prolong its t_{max} [31]. There is also evidence for an effect of time of administration on the bioavailability of verapamil. Hla *et al.* [32] gave a single 80 mg dose of verapamil to each of eight healthy volunteers at 4 AM, 8 AM, noon, 4 PM, 8 PM and midnight and found significantly higher plasma concentrations after 8 AM and noon administration than at any other time.

Distribution

After intraperitoneal administration to rats, and intravenous administration to dogs, verapamil is found in relatively high concentrations in liver, kidney, lungs and heart tissue [33]. In normal subjects, verapamil is widely distributed throughout body tissues. It crosses the placenta and is present in umbilical vein blood at delivery. Verapamil is distributed into milk, reaching concentrations in breast milk similar to those in maternal plasma in some patients. The apparent volume of distribution after i.v. drug administration in normal humans ranges from 162 to 380 L [34-35].

Verapamil is approximately 90% bound to plasma proteins in man. This binding is independent of concentration over a range of 50 to 1500 ng ml^{-1}. Of the protein bound fraction, 60% involves albumin and the remainder binds to α_1-acid glycoprotein [36]. *In* vitro studies revealed that plasma protein binding of verapamil increases with increase in α_1-acid glycoprotein [36]. Young *et al.* [37] reported a significant decrease in verapamil protein binding in the presence of therapeutic concentrations of several basic drugs (propranolol, diazepam, lidocaine and disopyramide) and an acidic drug (salicylic acid). Plasma protein binding of verapamil was not influenced by its major metabolite norverapamil in concentrations exceeding the verapamil concentrations by up to 26 times [38]. In post-operative patients and in patients on haemodialysis, plasma protein binding of verapamil was not different from healthy volunteers [38].

Metabolism

Verapamil is rapidly and almost completely metabolized by the liver in animals [39] and in man [40]. A hepatic extraction ratio of 0.8 was determined by direct measurement of plasma concentrations in hepatic arterial and venous blood [41]. Wide interpatient variations in drug metabolism are observed with verapamil, a finding consistent with drugs undergoing extensive first-pass extraction. N-dealkylation and O-demethylation are the main metabolic pathways. Although the O-demethylated metabolite of verapamil exhibits the same potency as the parent drug, its contribution to the overall pharmacological effect is negligible since this

metabolite is present in plasma as the glucuronide which has no pharmacological activity [40, 42]. Norverapamil is an N-demethylated metabolite, which has been found after oral verapamil administration, to have approximately 20% of the coronary vasodilation activity of the parent compound [43]. Norverapamil can reach plasma concentrations approximately equal to verapamil within 4-6 hours after the oral administration of verapamil.

Excretion

Verapamil is excreted as metabolites. Approximately 70% of the metabolites are excreted in the urine and 16% in the feces [40]. Only about 3-4% is excreted as unchanged drug in the urine. Plasma concentrations of verapamil appear to decline in a biphasic or triphasic manner both in animals [44] and man [35] following i.v. administration of the drug. The reported terminal elimination phase half-life for verapamil ranged from 2.7 to 4.8 hours [42]. Due to saturation of hepatic enzyme systems, the half-life can be increase after repeated oral administration. The systemic clearance of verapamil is very high suggesting that it is eliminating at a rate that is dependent on hepatic blood flow. Therefore, factors that change hepatic blood flow can alter its clearance [42].

Drug Interactions

Verapamil is currently used in the treatment of various cardiovascular diseases such as hypertension, angina, and cardiac arrhythmias, so it is likely that it

could be used concurrently with other drugs used in these disorders. The dependence of verapamil upon hepatic blood flow, hepatic metabolizing capacity and its high degree of protein binding provide several mechanisms whereby drug interactions may occur. Some of these have been reviewed [45-46].

Studies have shown that the coadministration of verapamil in patients receiving digoxin increased digoxin plasma concentration [47-48]. Verapamil reduced the total body clearance of digoxin by 39%, decreasing both renal (20% reduction) and non-renal (60% reduction) drug clearance [48-49]. The mechanism underlying the renal interaction, although not fully elucidated, appears to be suppression of tubular secretion of digoxin without reduction in glomerular filtration rate [49]. Although renal clearance accounts for approximately 75% of total body clearance, the decrease in non-renal clearance of digoxin is also important [50]. The greatest impact of the altered hepatic metabolism of digoxin would occur in patients with severe renal impairment, because these patients are more dependent on an unaltered hepatic metabolism for the drug.

Rahan *et al.* [51] reported a remarkable decrease in the bioavailability of verapamil when it was administrated with rifampicin. This finding is not surprising when the extensive first-pass metabolism is considered. Rifampicin is well-known as an inducer of hepatic enzymes and the enhanced enzyme activity associated with its use apparently allows for a virtual first-pass elimination of all absorbed verapamil.

The influence of cimetidine (a potent microsomal enzyme inhibitor) on the pharmacokinetics of verapamil has been studied by many investigators [52-53]. These investigators found 26% to 49% increase in the bioavailability of orally administered verapamil in the presence of cimetidine.

VERAPAMIL AND CHIRALITY

Verapamil has one center of asymmetry and is marketed as a racemate. However, considerable evidence indicates that the two enantiomers differ in their pharmacological potencies with the S-enantiomer being 10 to 20 times more potent than the R-enantiomer in terms of negative dromotropic effect on atrioventricular conduction in man [14] and in animals [54-55]. Qualitative differences in enantiomeric activity were also reported [56]. In the intact dog dominant antiarrhythmic activity of S-verapamil was found, but the excitability depressing properties of R-verapamil, against aconitine-induced ventricular arrhythmias *in vivo,* were described as an additional pharmacological activity [56]. In another study, both enantiomers of verapamil demonstrated equal antifibrillatory activities in acute regional myocardial ischemia in the perfused rat heart, but the racemic agent was more effective than either enantiomer alone suggesting multiple components of action [57].

The pharmacokinetics of verapamil enantiomers also differ from each other after intravenous and oral doses in both human and in some animals [58-63]. Cimetidine is shown to inhibit metabolism of S-verapamil to a greater extent than

R-verapamil [64]. Recent studies have also shown age- and gender-related differences in the stereoselective disposition of verapamil [65-68].

VERAPAMIL STEREOSELECTIVITY AND INPUT RATE

Verapamil has a relatively short half-life ($t_{1/2}$) [69]. In an attempt to obtain a clinically acceptable duration of response, clinicians have utilized various controlled-released (CR) preparations of verapamil. The use of a CR product overcomes the relatively short t½ of verapamil while maintaining blood pressure control for a longer period of time. However, the administration of CR products must be carefully studied as it is likely that the first-pass metabolism of verapamil enantiomers is dependent upon the rate of verapamil delivery to the liver which, in turn, is dependent on the delivery of drug via hepatic blood flow once the drug has reached the portal circulation. It is possible that, as a result of the formulation administered, the same patient may present with differing responses to the same daily dose of medication.

Woodcock *et al* [70] compared data published by several authors and postulated that the extent of verapamil first pass metabolism was determined by Michaelis-Menten kinetics. They also indicated that absolute bioavailability increased when the drug exposed to the liver upon first pass exceeded the ability of the liver to extract the drug. Comparing equal doses of verapamil (CR 240 mg once daily *vs* IR 80 mg three times per day), Harder *et al.* [71] found the IR formulation to be more potent (in terms of PR interval prolongation) than the CR

formulation. They explained this by suggesting that oral input rates of racemic verapamil can affect the enantioselective first-pass metabolism, consequently altering the proportion of the more active S-verapamil present in the total verapamil. Using computer simulations Mehvar [72-73] hypothesized that the rate of drug entry into the portal blood from the gastrointestinal tract can affect the ratio of blood concentration of its enantiomers. Later Mehvar and Reynolds [74], using isolated perfused rat liver studied the input rate-dependency of verapamil kinetics [74]. They found that a two-fold increase in the input rate, resulted in a significant increase in the hepatic availabilities of verapamil enantiomers. This increase was more pronounced for R-verapamil resulting in a significant decrease in the S:R availability ratio. Recently Longstreth *et al.* [91] and Karim *et al.* [75] found significantly different S:R ratios of verapamil in human plasma from oral formulations that produced substantially different input rates.

VERAPAMIL AND β-BLOCKERS

Several studies have documented the enhanced benefits of a combination regimen of verapamil/β-blockers when compared with the efficacy of either drug alone in the treatment of chronic stable angina [76-78]. In two different studies Leon *et al.* [76] and Subramanian *et al.* [77] found no significant adverse effects after the coadministration of verapamil and propranolol. In contrast, studies done by other investigators [78-80] using propranolol or metoprolol with verapamil, reported severe adverse effects, including heart failure. Although

pharmacodynamic interactions were assumed to be the cause of these adverse reactions, other studies suggested that the combination of β-blockers with verapamil may result in pharmacokinetic interactions [81]. Many β-blockers affect the hepatic blood flow [82] and can alter the systemic clearance of verapamil. Furthermore, some β-blockers are capable of inactivating P-450 isozyme [83]. As verapamil is metabolized by the liver P-450 isozymes [84-86], it is possible that some β-blockers can interfere with this pathway.

RATIONALE FOR STUDY

Verapamil is frequently used in the treatment of cardiovascular disorders such as hypertension, angina pectoris, and supraventricular tachycardia. Verapamil is administered as a racemate; the two enantiomers differ considerably in their pharmacokinetic and pharmacological properties. Various IR and CR formulations of verapamil are available on the market. The release rate of verapamil (input rate) from CR formulation is expected to be different from that of IR formulation. As suggested by some investigators [72-74], differences in the input rates can change the S:R ratio of verapamil in plasma. As the pharmacological properties of S- and R-verapamil are unequal, the change in the S:R ratio can produce different therapeutic outcomes. A study of the disposition of verapamil enantiomers after IR and CR administration, could clarify the effect of input rate on S:R ratio.

Verapamil and β-blockers are used in the treatment of various forms of angina pectoris. This combination provides synergistic efficacy in patients with

chronic stable angina who remain symptomatic with either agent alone. Severe adverse effects have been reported in a large number of patients receiving combination therapy which may be the result of a pharmacokinetic interaction. Some of the β-blockers have a tendency to decrease the hepatic blood flow [82], upon which verapamil metabolism is critically dependent. It has also been reported that the more lipid soluble β-blockers can inhibit liver enzymes [87]. Because verapamil is highly cleared by the liver, this inhibition can interfere with the metabolism of verapamil enantiomers. Therefore, it is important to study the effect of β-blockers on the pharmacokinetics of verapamil enantiomers. Furthermore, due to the risk of severe adverse reactions, including heart failure, this study should be done in an animal model.

Selection of an Animal Model for the Interaction Study

Sprague-Dawley rats were used to study the effect of a β-blocker on the pharmacokinetics of verapamil enantiomers. There is no information in the literature regarding the disposition of verapamil enantiomers in the rat. Non-stereoselective studies, however, suggested that disposition and elimination characteristics of verapamil were comparable to those in humans. The pharmacokinetics of verapamil enantiomers investigated in the rat prior to the drug-drug interaction study.

Selection of a Suitable β-blocker for the Interaction Study

[2]Metoprolol is a β-blocker which lacks intrinsic sympathomimetic activity [88]. It is used extensively in the treatment of hypertension and ischaemic heart disease. Although Metoprolol is administered as the racemate, most of the β-blocking activity resides with the S-enantiomer [89]. Differences in the kinetics of the two isomers have also been reported [90].

Metoprolol was selected for the following reasons.

1. Like verapamil, metoprolol is extensively and completely metabolized by the rat liver.
2. Metoprolol does not have intrinsic sympathomimetic activity (ISA). There is the possibility that it will decrease hepatic blood flow more than β-blockers which possess ISA.

[2] Metoprolol is referred to racemic Metoprolol through the thesis, unless specified otherwise.

Hypotheses

1. The dispositions of verapamil and norverapamil are stereoselective after the administration of racemate to humans and the systemic availability of the more active S-verapamil is lower than R-verapamil.

2. The extent of stereoselectivity (i.e., enantiomeric ratio) is dependent upon the formulation (e.g., immediate release *versus* control release).

3. The dispositions of verapamil is stereoselective in the Sprague-Dawley rat.

4. The pharmacokinetics of verapamil enantiomers are altered when given with metoprolol.

Objectives

1. Establish suitable stereospecific methods for quantification of verapamil and norverapamil enantiomers.

2. Delineate the pharmacokinetics of verapamil and norverapamil enantiomers in healthy volunteers following oral administration of immediate- and controlled-release formulations of the racemate.

3. Delineate the pharmacokinetics of verapamil enantiomers after intravenous and oral administration of the racemate in rat.

4. Delineate the pharmacokinetics of verapamil enantiomers in the rat, in the presence and absence of metoprolol.

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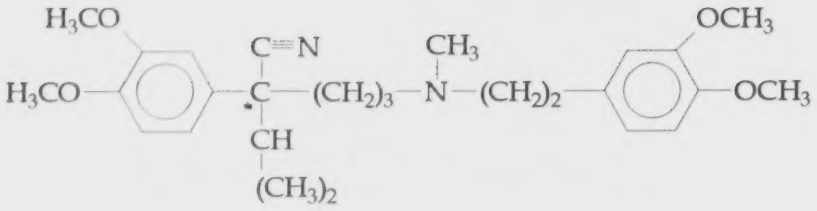

Figure 1-1. Chemical structure of racemic verapamil. Asterisk (*) denotes the chiral carbon.

2 STEREOSPECIFIC ASSAYS

I. MICROWAVE-FACILITATED PRE-COLUMN DERIVATIZATION AND HPLC ANALYSIS OF VERAPAMIL AND NORVERAPAMIL ENANTIOMERS IN HUMAN PLASMA

INTRODUCTION

Hypertension, a leading risk factor in the development of cardiovascular disease [1], is often treated with β-adrenergic blocking drugs or with calcium channel antagonists. A significant number of these drugs exist as racemates. Therefore, considerable attention has been given to the development of stereospecific analytical techniques [2-8] which may be applied to describing the pharmacokinetics and dynamics of a particular drug.

A number of analytical methods for verapamil have been previously described [9-17]. However, several of the assays reported have not been stereospecific. Stereospecific assays for verapamil, however, are necessary as the S- enantiomer of verapamil possesses significantly greater negative inotropic, chronotropic

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and dromotropic potencies, compared with the R-enantiomer [18]. Chiral column high-performance liquid chromatographic (HPLC) methods for the analysis of verapamil and norverapamil (Fig. 2-1) enantiomers have been reported on β-cyclodextrin [19], α_1-acid glycoprotein [20,21], ovomucoid [22,23], cellulose [24] and derivatized amylose [25] columns. Some of these assays require comparatively elaborate systems consisting of several pumps and coupled achiral-chiral columns with a column-switching procedure. More recently, Dethy and co-workers have reported stereospecific analysis of verapamil and norverapamil by capillary electrophoresis [26]. In this paper, we report a sensitive HPLC assay for the enantiomers of verapamil and its major metabolite, norverapamil using pre-column derivatization with a unichiral reagent.

MATERIALS AND METHODS

Chemicals

Racemic verapamil hydrochloride, racemic norverapamil hydrochloride, and pure enantiomers of verapamil and norverapamil reference samples were provided as gifts from Searle (Skokie, IL, USA). Internal standard (I.S.) pindolol reference sample was provided as a gift from Sandoz (Dorval, Canada). The derivatizing reagent, (-)-menthyl chloroformate ((-)-MCF) and ethanolamine (EOA) were purchased from Aldrich Chemical Company, Inc. (Milwaukee, WIS, USA). Triethylamine (TEA) was obtained from Fisher Scientific (Fair Lawn, NJ,

USA). Solvents including methyl *t*-butyl ether (MTBE), isooctane, acetonitrile, methanol and water were obtained from BDH (Toronto, Canada) and were HPLC grade.

Apparatus and Chromatography

Samples were vortex-mixed using a Vortex Genie 2 mixer (Fisher Scientific, Edmonton, Canada), centrifuged with a Dynac II centrifuge (Becton Dickinson, Parsippany, NJ, USA), and microcentrifuged with a Beckman Model E microcentrifuge (Palo Alto, CA, USA). Solvents were evaporated using a Savant Speed Vac concentrator-evaporator (Emerston Instruments, Scarborough, Canada). The HPLC system consisted of a Model 590 pump and a Model 712 WISP autosampler (Waters Scientific, Mississauga, Canada). Fluorescence detection (Millipore Model 470, Waters) was set at 220 and 330 nm for excitation and emission, respectively, for the first 16 mins, and at 280 and 320 nm, respectively for the remainder of the run [the detector was programmed to optimize detection of both internal standard and analyte]. A Millennium 2010 Workstation, version 2.00 (Waters Scientific, Mississauga, Canada) was used to record and process the chromatograms. The column consisted of two coupled C8-Novapak, 4 mm diameter cartridges housed in an 8 cm x 10 cm module with extender kit (Waters Scientific, Mississauga, Canada). The mobile phase was methanol-water (80:20, v/v) pumped at a flow-rate of 2.0 ml/min. To facilitate the derivatization reaction for verapamil, a microwave oven (Quasar Model MQS

1103H, Matsushita Electric of Canada Ltd., Mississauga, Canada) was obtained from a local commercial supplier.

Standard Solutions

Stock solutions of verapamil and norverapamil were prepared in water to yield final concentrations of 50 μg/ml (of each enantiomer) of base. Immediately prior to sample preparation, additional solutions were made by dilution with water. Other sets of verapamil and norverapamil stock solutions were prepared in acetonitrile to yield the same final concentrations as the aqueous solutions. These latter solutions were used in determination of extraction and derivatization yields. The internal standard, racemic pindolol, was prepared as a stock solution in water-methanol (90:10, v/v) to a concentration of 50 μg/ml (of each enantiomer). A stock solution of (-)-MCF was prepared as a 1:10 (v/v) solution in acetonitrile. Other stock solutions included saturated sodium carbonate, MTBE-isooctane (75:25, v/v), TEA-acetonitrile (1:40, v/v) and EOA-acetonitrile (1:50, v/v).

Sample Preparation

Drug-free human plasma (1.0 ml) was spiked with aqueous concentrations of 0, 10, 50, 100, 250, and 500 ng/ml each of norverapamil and verapamil (as base). To this plasma was added 100 μl of I.S. and 50 μl of saturated sodium carbonate solutions. Samples were then extracted with 4 ml of a mixture of MTBE-isooctane 75:25, v/v). The resultant mixture was vortex-mixed for 30 s

and centrifuged for 5 mins at 1800 x *g*. The organic layer was drawn off and separated into two equal portions (portions A and B) for independent analysis of norverapamil and verapamil, respectively. Both portions were evaporated to dryness using a Savant Speed Vac concentrator-evaporator. To the residues, were added 100 µl each of acetonitrile solutions of TEA 1:40 (v/v) and (-)-MCF 1:10 (v/v).

To portion A (S and R- norverapamil assay), 50 µl of EOA in acetonitrile 1:50 (v/v) was added to the mixture, 30 s after the addition of (-)-MCF. The mixture was vortexed for 30 s and the solvent evaporated. The residue was reconstituted with 200 µl of mobile phase and the solution transferred to a polypropylene microcentrifuge tube. After microcentrifugation for 2 min, a 120 µl portion was placed into a glass microinsert and an aliquot of 40-100 µl was injected onto the HPLC.

To portion B (S and R- verapamil assay), after addition of (-)-MCF, the mixture was microwaved at full power (voltage: 4 kV DC, frequency: 2450 Mhz) for 2 mins. After 2 min, 50 µl of the EOA in acetonitrile 1:50 (v/v) solution was added. The mixture was vortexed for 30 s and the solvent evaporated. The remaining residue was reconstituted with mobile phase and the solution transferred to a polypropylene microcentrifuge tube. After microcentrifugation (2 mins) a 120 µl portion was placed into a glass microinsert and an aliquot of 40-100 µl injected onto the HPLC.

Quantitation

Calibration curves were constructed by plotting the peak-area ratios (verapamil or verapamil/I.S.) obtained *versus* the corresponding enantiomer concentration added to plasma. The first eluting peak of the I.S. was used for quantification of the peak-area ratio. Results are reported as mean ± S.D. The use of $1/x^2$ weighting is applied in all calculations of calibration curves, where x is the added enantiomer concentration of verapamil and norverapamil.

Extraction Yield

Solutions (n = 3) containing 50, 100 and 250 ng/ml (of each enantiomer) of norverapamil and verapamil in acetonitrile were added to clean, dry, glass tubes and evaporated. After addition of 1.0 ml plasma, verapamil and norverapamil were extracted as previously described except that exactly 3.0 ml of the organic layer was transferred to a clean dry glass tube and evaporated to dryness. To compare extracted *versus* unextracted samples, an equivalent amount of verapamil and norverapamil (using solution 2) was added to another set of glass tubes containing only 4 ml extraction solvent (n = 3); 3 ml was transferred to a clean, dry, glass tube and evaporated to dryness. The peak areas of extracted *versus* unextracted verapamil and norverapamil samples were compared under identical chromatographic conditions. A previously reported non-stereospecific assay was used for the extraction yield determinations [9].

Derivatization Yield

Solutions (*n*= *3*) containing 50, 100 and 250 ng/ml (of each enantiomer) of norverapamil and verapamil in acetonitrile were added to clean, dry, glass tubes and evaporated. After derivatization using (-)-MCF, in the absence of IS, samples were chromatographed using a nonstereospecific HPLC assay [9]. The derivatization yields were determined by measuring the norverapamil and verapamil peak areas remaining after addition of (-)-MCF.

Accuracy and Precision

Drug-free plasma was spiked with norverapamil and verapamil at six different enantiomer concentrations (n = 9 for each concentration) over the range 10-500 ng/ml. Accuracy was assessed by determining the concentration of drug measured in each sample relative to the known concentration added and was expressed as the percent analytical recovery (% AR). Precision was calculated by determining percent inter-day coefficient of variation (% CV).

RESULTS AND DISCUSSION

A number of different HPLC stereospecific techniques [19-26] for the analysis of verapamil and norverapamil enantiomers in biological samples have been utilized. Several of these techniques require the use of chiral columns [22-

25]. Additionally, these assays may require comparatively elaborate systems consisting of more than one pump and achiral-chiral columns with column-switching procedures [19-21]. As an alternative to coupled, achiral-chiral columns, the present report utilized microwave-facilitated, pre-column derivatization with a unichiral reagent followed by resolution on an achiral reversed-phase column.

The most frequently reported methods of formation of diastereomers with chiral drugs such as verapamil which have tertiary amines have been limited to compounds with additional reactive functional groups [27-31], which verapamil lacks. There are a number of reports on the use of (-)-MCF for the derivatization of secondary alcohols [32] and secondary amines [33-36]. However, reactions between tertiary amines and chloroformates have generally been used as the first step in a two-step procedure that results in N-dealkylation. The first step results in the formation of a carbamate derivative [37] that can further be hydrolyzed to form a secondary amine. Subsequently, addition of a unichiral isocyanate then forms the urea derivative. These reactions require relatively lengthy chemical procedures and are not generally suitable for routine analysis of plasma samples. Prakash *et al.* reported the formation of stable carbamate diastereomers by the direct reaction of (-)-MCF and tertiary amines in the presence of heat [38]. In our experience we found that (-)-MCF reacted rapidly with norverapamil at room temperature to form stable carbamate diastereomers whereas the reaction with verapamil at room temperature was slow. By heating however, the methyl group was more easily

cleaved and verapamil reacted rapidly with (-)-MCF forming the same carbamate diastereomer (Fig. 2-1) as was formed for norverapamil.

Derivatization of verapamil with (-)-MCF by heating at 60^0 C required a reaction time of approximately 2 h. This period was reduced to 30 min by heating the sample to 100^0 C. With a large number of samples, however, this requirement for heat may present some practical difficulties (e.g., stability, homogeneous heating). A microwave oven was used, as it seemed reasonable to expect that a combination of heat and microwave energy might be an effective alternative to conventional oven heating [39-40]. Various reaction times were tested at low, medium, and high microwave settings. A time of 30 sec at the high setting (voltage: 4 kV DC, frequency: 2450 Mhz) was found to provide a derivatization which was >95% complete. This was tested using a non-stereospecific assay [9] which did not detect underivatized verapamil after microwave-assisted derivatization of the sample with (-)-MCF.

Figure 2-2 depicts chromatograms of blank plasma, plasma samples spiked with 100 ng of each norverapamil enantiomer, plasma spiked with 200 ng/ml of each (verapamil plus norverapamil) enantiomer, and a 3 h norverapamil plasma sample from a healthy subject dosed with a single oral 80-mg verapamil tablet. Peaks corresponding to S and R- verapamil (or norverapamil) eluted at approximately 26.5 and 27.4 min, respectively (R_s = 1.65, α = 1.06). Peaks corresponding to S- and R-I.S. eluted at approximately 10.5 and 11.5 min, respectively. The order of elution was determined by chromatography of the pure

enantiomers. Blank plasma samples were free of any interfering peaks. Calibration curves for S and R- verapamil were typically described by $y = 0.053 + 0.012\ (x)$, and $y = 0.067 + 0.012\ (x)$, where y is the enantiomer concentration (ng) and x is the peak area ratio. The peak-area ratios and the added concentrations displayed an excellent linear relationship with correlation coefficient, which were typically > 0.996 for derivatized enantiomers. The use of $1/x^2$ weighting was included in all calibration curves as this tended to bias low concentrations but still provided acceptable accuracy at the highest concentrations.

The results in Tables 2-1, 2-2 and 2-3 describe accuracy and precision of the method. Both accuracy and precision values throughout the concentration range were within approximately 15% of the expected values.

The extraction yield of verapamil from plasma, as indicated by comparing extracted *versus* unextracted samples, was found to be at least 80% for both enantiomers. Although the extraction was not quantitative, it provided adequate reproducibility and sensitivity to quantitate clinical samples.

In conclusion, a sensitive and stereospecific reversed-phase HPLC method for the analysis of verapamil and norverapamil enantiomers was reported, using a an optically pure derivatizing agent. The derivatization procedure was optimized by making use of a microwave-facilitated reaction. The assay proved to be valid for the determination of verapamil and norverapamil in clinical samples.

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Table 2-1: Accuracy and Precision Data for Norverapamil in Human Plasma

Concentration Added (ng/ml)[a]	Coefficient of Variation (%CV)		Analytical Recovery (%AR)	
	S-NV	R-NV	S-NV	R-NV
10	6.3	3.4	102	100
50	15.2	12.6	103	97.8
100	4.8	9.4	101	99.3
250	11.1	7.5	94.2	92.0
500	7.5	6.5	110	111

[a] *n=9 (3 sets for 3 days).*

NV= norverapamil

Table 2-2: Accuracy and Precision Data for Verapamil plus Norverapamil

Concentration Recovery Added (ng/ml)[a]	Coefficient of Variation (%CV)		Analytical (%AR)	
	S-NV + V	R-NV + V	S-NV + V	R-NV + V
20	1.5	1.1	101	101
100	9.7	6.3	97.6	91.8
200	11.2	11.9	100	99.6
500	8.7	9.4	94	102
1000	6.9	7.7	107	106

[a] concentration added corresponds to equal amounts of norverapamil and verapamil in plasma.

NV = norverapamil

V = verapamil

Table 2-3: Accuracy and Precision Data for Verapamil

Concentration Recovery Added (ng/ml)[a]	Coefficient of Variation (%CV)		Analytical (%AR)	
	S-V [a]	R-V [a]	S-V [a]	R-V [a]
10	6.7	2.7	97.5	101
50	16.3	16.2	100	85.7
100	14.2	13	95.8	91.7
250	17.3	17.8	96.4	101
500	12.9	16.8	103	101

[a] calculated as (NV plus V) minus NV.
V = verapamil, NV = norverapamil

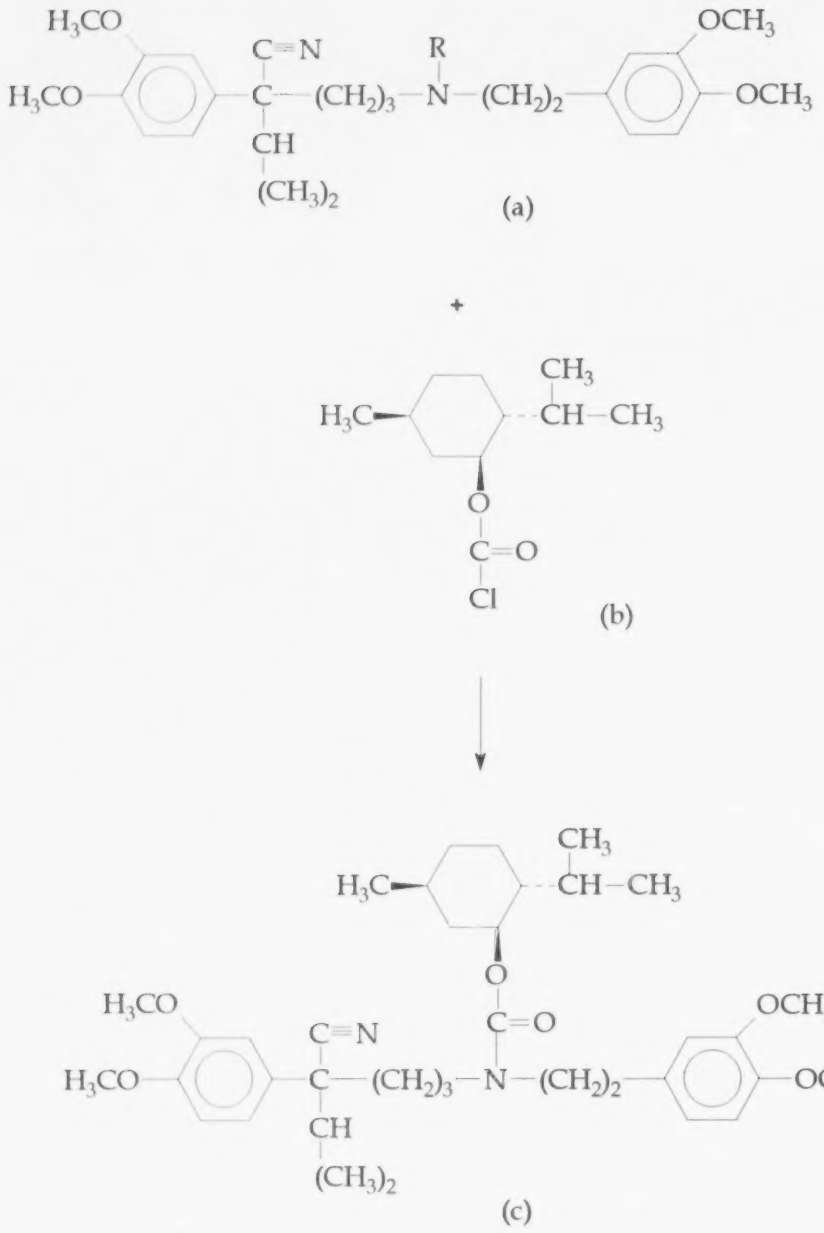

Figure 2-1. Chemical structures of (a) racemic verapamil; (b) (-)-MCF; and (c) derivatized verapamil.
R=CH_3, verapamil
R=H, norverapamil

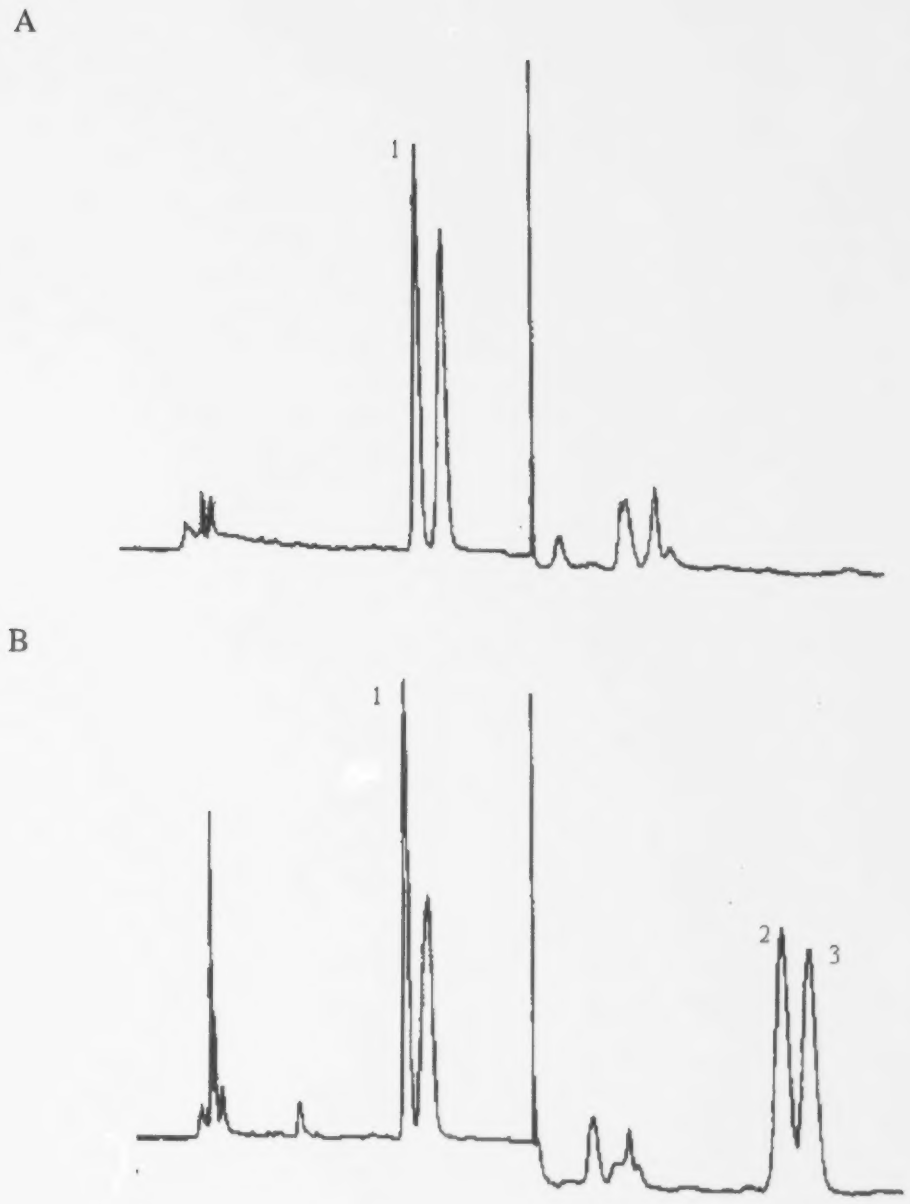

Figure. 2-2: Chromatograms of (A) blank human plasma, (B) human plasma spiked with 100 ng/ml (of each enantiomer) of NV. For Peak identification please see figure 2-2 on the next page

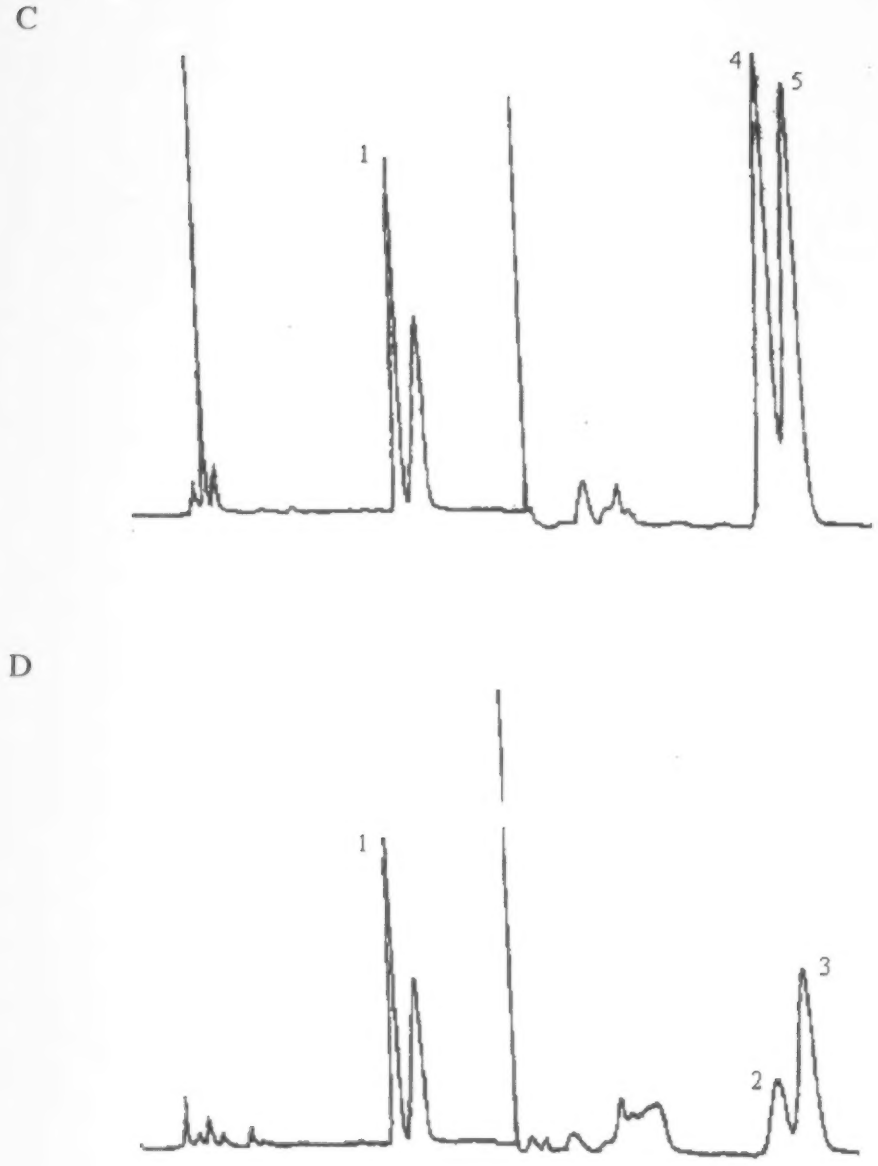

Figure. 2-2: Chromatograms of (C) human plasma spiked with 100 ng/ml of V plus 100 ng/ml NV (each enantiomer), (D) human plasma sample of NV taken 6 h after oral administration of a single 80 mg dose of an immediate release V. Peak identification: (1) I.S. (pindolol) (2) S-NV, (3) R-NV, (4) S-(V plus NV), (5) R-(V plus NV).

II STEREOSPECIFIC HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY OF VERAPAMIL AND NORVERAPAMIL IN HUMAN PLASMA USING A CHIRAL STATIONARY PHASE

INTRODUCTION

Verapamil, is a calcium channel blocking drug which is marketed as the racemate. Verapamil is extensively metabolized to the active metabolite norverapamil which also contains an asymmetric carbon. The S-enantiomers of both verapamil and norverapamil have virtually all of the desired pharmacologic activities (eutomers) whereas the R-enantiomers are regarded as being much less active (distomers) [1]. As the dispositions of the enantiomers of verapamil and norverapamil are stereoselective in man [2], and in some animal models [3], the measurement of verapamil and norverapamil in biological materials must be stereospecific.

Chiral column high-performance liquid chromatographic (HPLC) methods for the analysis of verapamil and norverapamil enantiomers have recently been reported on β-cyclodextrin [4], α_1-acid glycoprotein [5-6], ovomucoid [7-8],

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and cellulose [9] columns. Some of these assays require comparatively elaborate systems consisting of several pumps and sequential achiral/chiral columns with a column-switching procedure. Although Shibukawa *et al.* [10] reported the use of amylose tris (3,5-dimethylphenyl carbamate) to separate verapamil and norverapamil enantiomers, their method required an additional preceding achiral column to effectively separate the enantiomers from the I.S. Recently Dethy and co-workers have reported the stereospecific analysis of verapamil and norverapamil by capillary electrophoresis using chiral mobile phase additive [11]. More recently our laboratory has developed a microwave-facilitated pre-column derivatization HPLC analysis of verapamil and norverapamil enantiomers in human plasma [12].

In this paper, we describe the separation of verapamil and norverapamil enantiomers using a commercially available chiral column. This method does not require derivatization or achiral chromatography prior to chiral column chromatography.

EXPERIMENTAL

Chemicals

Racemic verapamil hydrochloride, racemic verapamil hydrochloride, and pure enantiomers of verapamil and norverapamil reference standards were provided as gifts from Searle (Skokie, IL, USA). Internal standard, (+)-naproxen

chloride, (I.S.) was synthesized in our laboratory by reacting thionyl chloride with (+)-naproxen and purified by repeated recrystallization. Thionyl chloride and (+)-naproxen were obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). Triethylamine (TEA) was obtained from Fisher Scientific (Fair Lawn, NJ, USA). Reagent-grade sodium carbonate was obtained from BDH (Toronto, Ontario, Canada). HPLC-grade hexane was obtained form Baxter Healthcare Corporation (Muskegon, MI, USA). Analytical-grade methyl *t*-butyl ether (MTBE), isooctane, isopropyl alcohol and HPLC-grade water were obtained from Mallinckrodt (Paris, KT, USA).

Chromatography

The HPLC system consisted of a Model 590 pump, Model 712 Wisp autosampler, Model 470 fluorescence detector, and Millennium (version 2.1) computer data acquisition and integration software (Waters Scientific, Mississauga, Ontario, Canada). The chromatography utilized a 4.6 x 250 mm amylose carbamate-packed chiral column (Chiralpak AD, Chiral Technologies, Exton, PA, USA). The mobile phase consisted of hexane:isopropyl alcohol:triethylamine (90:9.9:0.1 v/v) pumped at a flow rate of 1.2 ml/min. Fluorescence detection was set at 272 nm and 317 nm for excitation and emission respectively.

Standard Solutions

Stock solutions of verapamil and norverapamil were prepared in HPLC grade water to yield final concentrations of 50 μg/ml of each enantiomer (as base). Immediately prior to sample preparation, additional solutions were made by dilution with water. Another set of verapamil and norverapamil stock solutions were prepared in methanol to yield the same final concentrations as the aqueous solutions. These latter solutions were used in determination of extraction efficiency. The internal standard, (+)-naproxen chloride, was prepared as a stock solution in methanol to a concentration of 100 μg/ml. The lake of any interfering peak and excellent reproducibility of the assay indicated that (+)-naproxen chloride solution was quite stable. Other stock solutions included saturated sodium carbonate and methyl *t*-butyl ether:isooctane (75:25, v/v). The I.S., verapamil and norverapamil stock solutions were stored at 5 °C.

Sample preparation

Drug-free human plasma samples (1.0 ml) were spiked with aqueous concentrations of 0, 5, 10, 50, 100, 250, and 500 ng/ml each of norverapamil and verapamil (both as base). This was followed by addition of 0.5 μg of I.S., 50 μl of saturated sodium carbonate solution and 4 ml of MTBE:isooctane (75:25, v/v). Samples were then vortex-mixed for 30 s (Vortex Genie 2 mixer, Fisher Scientific, Edmonton Alberta, Canada), and centrifuged at 1800 x *g* for 5 minutes (Dynac II, Becton Dickinson, NJ, USA). The organic layers were transferred to clean test

tubes, and evaporated to dryness (Savant Speed Vac concentrator-evaporator, Emerston Instruments, Scarborough, Ontario, Canada). The remaining residues were then reconstituted with 200 µl of mobile phase, vortexed for 10 s, and aliquots of 100 µl were injected into the HPLC.

Extraction Efficiency

Methanol stock solutions containing 50, 100 or 250 ng of each verapamil and norverapamil enantiomer ($n = 3$) and 0.5 µg of I.S. were added to clean, dry glass tubes and evaporated to dryness. After addition of 1.0 ml drug-free plasma and 50 µl of saturated solution of sodium carbonate to each tube, samples were extracted with 4 ml MTBE:isooctane (75:25, v/v). The tubes were vortexed for 30 s and centrifuged at 1800 x g for 5 minutes. Two ml of the organic layer were then transferred to clean tubes, evaporated to dryness, reconstituted and chromatographed. To compare these samples with those that were not extracted, another set of tubes containing the above amounts of drug were prepared and evaporated without the addition of plasma and subsequent extraction procedure. Peak areas of extracted drug *versus* one-half unextracted peak areas were compared under identical chromatographic conditions.

Quantitation

Weighted (1/x) calibration curves for verapamil and norverapamil in plasma were obtained by plotting the peak-area ratios (verapamil or norverapamil/I.S)

after extraction and analysis *versus* the corresponding concentrations. Back-calculated concentrations of verapamil and norverapamil enantiomers were generated from the regression curve. Results are reported as mean ± standard deviation (S.D.).

Accuracy and Precision

Drug free plasma was spiked with racemic norverapamil and verapamil to give enantiomer concentrations ranging from 5 to 500 ng/ml (n=9). Accuracy, expressed as the percent error (%error), was determined by comparing the concentration of drug measured in each sample relative to the known amount of each enantiomer added. Precision, expressed as percent coefficient of variation (%C.V.), was determined by back-calculation of concentrations from the respective calibration curves (n=9).

RESULTS AND DISCUSSION

Separation of the enantiomers of verapamil and norverapamil was achieved using a commercially available Chiralpak AD column. Figure 2-3 depicts representative chromatograms obtained from blank plasma as well as a spiked plasma sample (100 ng/ml each of verapamil and norverapamil enantiomer). The peaks corresponding to verapamil enantiomers eluted at approximately 6.5 and 7.5 minutes and peaks corresponding to norverapamil enantiomers eluted at approximately 11.7 and 13 minutes. The order of elution was determined by

chromatography of the pure enantiomers which indicated that the first and second eluting peaks (verapamil or norverapamil) corresponded to the S- and R-enantiomers, respectively. This elution order was the same as that reported by Shibukawa *et al.* [10] who originally reported the use of an amylose carbamate column in an achiral/chiral HPLC system. Shibukawa *et al.* [10] initially used an amylose carbamate column, but the peak corresponding to the internal standard, (+)-glaucine, co-eluted with the peak corresponding to R- verapamil. To separate these peaks from one another they used an achiral diol silica column preceding the chiral column. In our experience we observed that by using (+)-naproxen chloride as I.S an additional achiral column was not required since the corresponding peak eluted at least 2 minutes prior to those due to the verapamil enantiomers (Fig. 2-3). The resolution (R) and selectivity (α) values for the peaks corresponding to verapamil and norverapamil enantiomers were R_s=1.48, α=1.40 and R_s=1.19, α=1.14 respectively.

Tables 2-3 and 2-4 summarize the accuracy and precision data for verapamil and norverapamil respectively. At the lowest calibration concentration of 5 ng/ml, the coefficient of variation (%C.V.) was less than 3%. Over the entire concentration range, the %C.V. of the method never exceeded 9%. In addition, accuracy was within approximately 8.5% of the expected value. The mean extraction yield was greater than 80% under the conditions stated for extraction. The total time for the analysis of verapamil and norverapamil enantiomers was within 14 minutes at ambient temperatures. No attempts were made to shorten the

run-time (e.g. by altering operating temperatures) since sample throughput was adequate for our needs.

Since derivatization is not necessary this assay is more convenient than that previously reported by our laboratory [12]. Recovery of the pure enantiomers is an additional advantage. This method is also more convenient than other techniques in which two or more columns are required to separate verapamil and norverapamil enantiomers.

In conclusion, a sensitive, convenient stereospecific assay of verapamil and norverapamil was developed using a commercially available chiral column. The assay is valid for the determination of the enantiomers of verapamil and norverapamil with minimal sample preparation, thus enabling the routine use of this method for clinical studies.

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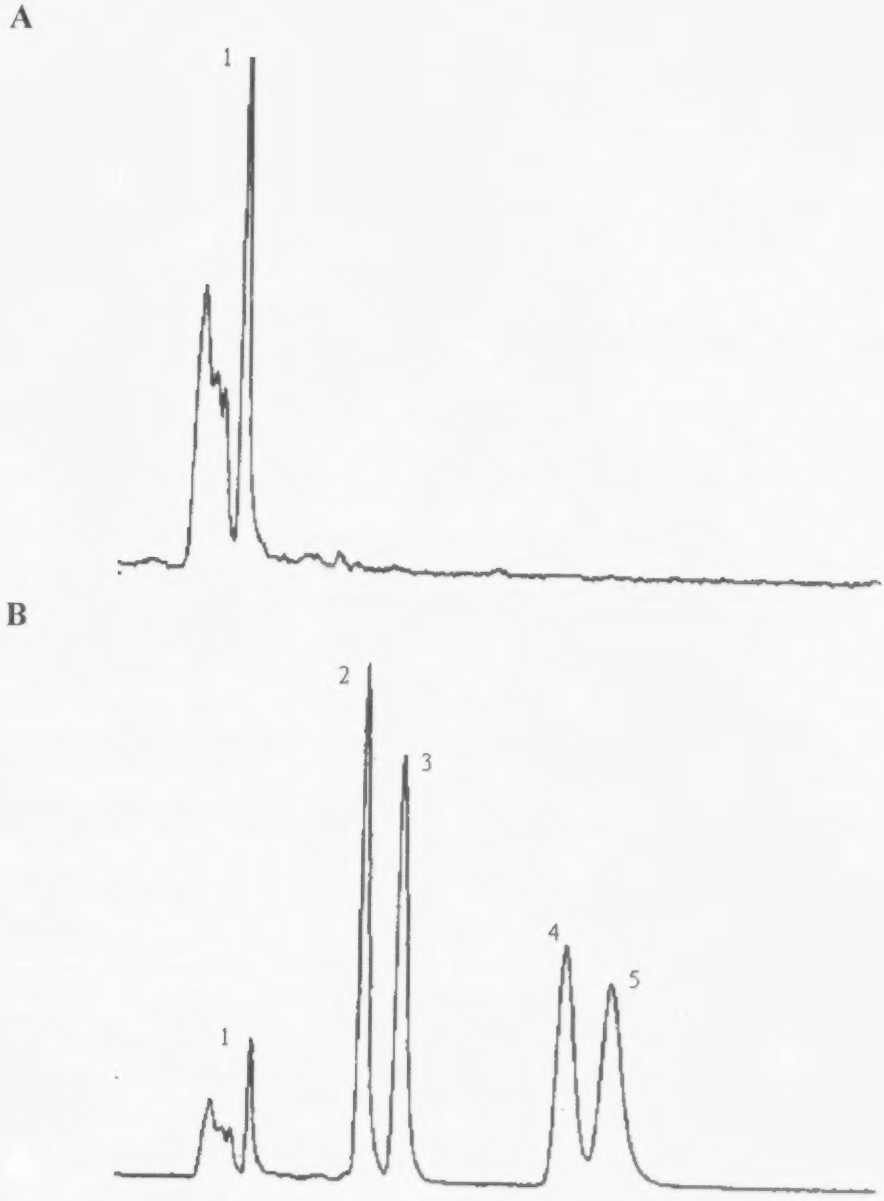

Fig 2-3. Chromatograms of (A) human plasma spiked with 5 μg/ml of I.S. (B) human plasma spiked with 100 ng/ml of each V enantiomer, 100 ng/ml of each NV enantiomer and 0.5 μg/ml of I.S. Peaks: 1=I.S. 2=S-verapamil; 3=R-verapamil; 4=S-norverapamil; 5=R-norverapamil.

Table 2-4. Accuracy and precision data for verapamil in human plasma (mean±SD).

Enantiomer concentration (ng/ml)[a]						
	Measured[b]		*Accuracy, error%*		*Precision, CV%*	
Added	*S-V*	*R-V*	*S-V*	*R-V*	*S-V*	*R-V*
5	5.3±0.1	5.3±0.1	6.4±1.5	6.2±1.7	1.4	1.7
10	9.3±0.7	9.5±0.7	4.9±4.8	1.0±0.6	7.4	7.4
50	55±4.9	52±3.1	8.6±6.3	6.3±3.6	9.0	6.0
100	98±4.9	98±5.1	4.1±3.1	4.8±2.4	5.0	5.2
250	254±4.9	257±5.1	4.9±3.1	5.0±2.4	2.0	2.0
500	473±15	478±15	6.9±3.5	5.8±2.4	3.2	3.2

[a] n = 9 (3 sets for 3 days)

[b] Reported as mean ± SD

V = verapamil , NV = norverapamil

Table 2-5. Accuracy and precision data for norverapamil in human plasma (mean±SD).

Enantiomer concentration (ng/ml)[a]						
	Measured[b]		*Accuracy, error%*		*Precision, CV%*	
Added	*S-NV*	*R-NV*	*S-NV*	*R-NV*	*S-NV*	*R-NV*
5	5.1±0.1	5.1±0.1	2.7±1.6	2.6±2.1	1.5	2.1
10	9.7±0.2	9.7±0.7	2.0±0.7	1.6±0.8	2.5	7.2
50	52±4.9	50±3.4	8.2±3.4	3.2±2.3	7.9	6.7
100	98±4.1	101±6.4	3.3±2.4	4.5±3.9	3.3	6.4
250	248±13	246±6.4	3.8±3.5	7.2±6.5	5.3	2.6
500	481±36	475±25	6.4±4.6	6.6±5.6	7.4	5.2

[a] n = 9 (3 sets for 3 days)

[b] Reported as mean ± SD.

V = verapamil , NV = norverapamil

3. PHARMACOKINETICS OF VERAPAMIL AND NORVERAPAMIL ENANTIOMERS FOLLOWING ADMINISTRATION OF IMMEDIATE AND CONTROLLED-RELEASE FORMULATIONS TO HUMANS: *EVIDENCE SUGGESTING INPUT-RATE DETERMINED STEREOSELECTIVITY*

INTRODUCTION

Verapamil is a calcium channel blocking drug used in the treatment of angina pectoris, hypertension and supraventricular tachycardia [1]. Although verapamil is manufactured and administered as the racemate, most of the calcium channel blocking activity resides with the S-enantiomer [2]. As enantiomers of many cardiovascular drugs, including calcium channel blockers, may exhibit stereoselective disposition, it is necessary to discern their respective time-courses. Additionally, as verapamil is extensively metabolized to a chiral metabolite, norverapamil, where the S-enantiomer also possesses desired pharmacologic activity [3-5], clinical laboratory measures must quantitate both the verapamil and norverapamil enantiomers in plasma. Although the literature is replete with reports describing the nonstereospecific disposition of verapamil (6-14), there is

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comparatively less published information regarding the stereospecific disposition of verapamil and norverapamil [15-20].

In an attempt to obtain a clinically acceptable duration of response and extent of blood pressure control, clinicians have utilized various controlled-release (CR) preparations of verapamil. Such preparations attenuate excessive peak-to-trough drug concentration fluctuations and thus may attenuate fluctuations in blood pressure response. Administration of a CR product overcomes the relatively short half-life (t½) of verapamil [3,5] while maintaining blood pressure control for a longer period of time. Administration of either immediate-release (IR) or CR products must be carefully studied as it is likely that the formation of norverapamil from verapamil is dependent upon the rate of verapamil delivery to the liver which, in turn, is dependent on the delivery of drug via hepatic blood flow once the drug has reached the portal circulation. It is conceivable that, as a consequence of the formulation administered, the same patient may present with differing responses to the same daily dose of medication. Determination of the enantiomeric dispositions of verapamil and norverapamil is therefore essential when conducting clinical investigations after administration of verapamil. Recently Abernethy and coworkers described the enantiomeric dispositions of verapamil after the administration of IR and CR products [21]. Although the findings of that report indicate that lower AUC values may be expected after CR dosing, the disposition of norverapamil was not delineated.

The present study examines the enantiomeric disposition of both verapamil and its major active metabolite, norverapamil, after administration of racemic verapamil to 11 healthy volunteers. Subjects received both formulations (IR and CR) in a cross-over fashion, on an identical mg-per-mg basis to evaluate the influence of the respective formulations on the disposition of verapamil and norverapamil.

EXPERIMENTAL SECTION

Subjects

Eleven healthy young volunteers (Table3-I) aged 20-55 years, within 15 per cent of ideal body weight, were entered into the study after giving written informed consent. The study protocol was approved by the Human Ethics Review Committee of the University of Alberta Hospital, Edmonton, Canada. All subjects were judged to be in good health and free from significant renal and hepatic impairment on the basis of a physical examination and screening laboratory tests that were conducted just prior to the study. Subjects were not receiving any other medications at the time of the study and refrained from alcohol and tobacco consumption prior to, and during, the study.

Study Design

Subjects received verapamil as either an IR or CR preparation on two separate occasions in a cross-over fashion with a minimum 1-week washout period between treatments. The IR treatment consisted of 80 mg verapamil administered orally three times daily (tid) for 7 consecutive days, whereas the CR treatment consisted of oral administration of 240 mg verapamil each morning for 7 consecutive days . The first dose of each treatment phase was administered in the Clinical Investigation Unit (CIU) at the University of Alberta Hospital. Subjects were asked to report any adverse side effects (headache, vertigo, bradycardia, palpitation etc.) during the study. Compliance was determined by counting the number of tablets remaining in each prescription vial after each formulation was tested for the one week duration. All prescription vials were returned to the CIU at the completion of both phases of the study.

At the completion of each study phase (Day 7), the patients returned to the CIU and remained in the clinic for the ensuing 24-h period. The seventh day dose was taken by the patients in front of CIU staff. During this time, blood samples were collected. A total of 10 ml of blood was collected at each sampling time via an indwelling forearm vein cannula immediately before, and at 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6 and 8 hours (for IR dosing; additional samples were taken at 20 and 24 h for CR dosing). Plasma was separated by centrifugation and stored at -20 C until immediately prior to the HPLC determination of verapamil and norverapamil enantiomers.

Protein Binding Study

Human blood samples were collected from four young male (mean 30 years; range 28-36) and four elderly male and female (mean age 67 years; range 64-71) subjects in heparinized tubes. The blood was centrifuged at 1850 x *g* for 20 mins and the plasma was separated and frozen at -20°C. The ultrafiltration method used for protein binding studies of verapamil and norverapamil was the same as that for sotolol [22] with a slight modification (37 °C water bath was used instead of 37 °C temperature controlled room). Briefly, ultrafiltration units equipped with low binding regenerated cellulose membranes of a molecular weight limit of 30,000 were used for ultrafiltration. Each filter unit consisted of a 1 ml septum-capped polypropylene tube containing a 1 ml sample cup with an ultrafiltration membrane tightly bound to its base. A 1 ml aliquot of each sample was placed in the sample cup of the filter unit and capped to prevent evaporation and pH changes. The filtration units were placed in a 37°C temperature-controlled water bath for 30 min and then centrifuged at 1850 x *g* for 1.5 h. A 300 µl aliquot of the resulting ultrafiltrate was removed for analysis of verapamil and norverapamil enantiomer content.

Pharmacokinetic Analyses

The areas under the plasma verapamil or norverapamil enantiomer concentration *versus* time curves (AUC) were determined using a Lagran computer program [23] for one dosing interval (0 to 8 h and 0 to 24 h for IR and CR,

respectively) on day 7 following each phase of the study. Oral clearance (Cl/F) was calculated by dividing the administered enantiomer dose with the corresponding AUC. The time to reach maximal plasma enantiomer concentration (Tmax) was determined directly from the data, as was maximal plasma enantiomer concentrations (Cmax). The fraction of the respective enantiomers reaching the systemic circulation (F) was not known, as only oral doses were administered. Fluctuation (expressed as percent) was calculated by taking the difference between Cmax and Cmin values throughout the interval and dividing by the Cmax.

Statistical Analyses

The pharmacokinetic parameters of the enantiomer disposition of verapamil and norverapamil were compared for both the IR and the CR products using a student's *t*-test for paired data. All tests were conducted at $\alpha = 0.05$. Data are expressed as mean ± SEM.

Quantification of verapamil and norverapamil

Concentrations of verapamil and norverapamil were determined via a stereospecific HPLC assay which was previously used in a dissolution study [30].

RESULTS

Figures 3-1 and 3-2 depict the mean plasma concentration *versus* time profiles obtained after administration of the IR and CR formulations for S- and R-

verapamil and for S- and R-norverapamil, respectively. These figures indicate that the dispositions of both verapamil and norverapamil are stereoselective. Figure 3 depicts the plasma disposition profiles for total (S plus R) verapamil and norverapamil concentrations following IR and CR administration. Efforts were also made to plot Tmax *vs* S:R AUC ratio or Tmax *vs* S:R Cmax ratio, but no correlation was found.

Pharmacokinetic parameters for S-and R-verapamil after IR and CR administration are shown in table 3-2. The AUC, Cmax and Cmin values of R-enantiomer are greater than S-enantiomer. The percent fluctuation for S-verapamil-CR, R-verapamil-CR and total verapamil-CR is greater than S-verapamil-IR, R-verapamil-IR and total verapamil-IR respectively. Table 3-3 describes the pharmacokinetic parameters of S-and R-norverapamil. The AUC and Cmax values of R-enantiomer are greater than S-enantiomer.

IR *versus* CR Administration

There was a general trend that concentrations of S-verapamil were reduced after CR administration, when compared with IR administration. Of the 11 subjects given CR formulation, only 5 had S-verapamil concentrations that were detectable for more than four time points during serial blood sampling. For these subjects the AUC was calculated from the available time points only. This difference, however, was not evident upon examination of total drug concentrations as S-verapamil constitutes only a minor fraction of overall verapamil blood concentration. Although a trend of greater AUC values for S-

norverapamil-IR, R-norverapamil-IR and total norverapamil-IR compared with S-norverapamil-CR, R-norverapamil-CR and total norverapamil-CR existed, due to high intersubject variations, the differences were not statistically significant. None of the other pharmacokinetic parameters were significantly different on comparing IR and CR formulations. There was, however, a statistically significant difference between peak-to-trough fluctuation of CR and IR products.

DISCUSSION

Protein Binding

The extent of binding of verapamil and norverapamil enantiomers to plasma proteins in different study groups is summarized in Tables 3-4 and 3-5. The binding of the R-enantiomers was greater than S-enantiomers in young and elderly humans. There were no significant difference between age groups. At the concentrations studied, norverapamil did not alter the binding of verapamil enantiomers (Table 3-4). The extent of verapamil and norverapamil binding measured in our study support the findings by others who studied non-stereospecific [23-24] and stereospecific [25, 26] binding in human. McGowan *et al.* [24] investigated the non-stereospecific protein binding of verapamil in human and found that approximately 91% was bound to plasma protein. In that study they also found that there was a good correlation ($r = 0.83$) between the percentage bound of verapamil and concentration of α_1-acid glycoprotein in the plasma. Keefe *et al.* [23] studied non-stereospecific verapamil protein binding in

normal subjects and in different groups of patients. In normal subjects, plasma protein binding of verapamil was 89.6% and was concentration-independent over a range of 35 to 1557 ng/ml. They also found that non-stereospecific plasma protein binding of verapamil was not affected by the addition of norverapamil in ratios of 1.2 to 26.3 (norverapamil/verapamil). These results were consistent with our stereospecific binding experiment of verapamil in the presence of norverapamil. In a recent study Johnson *et al.* [27] determined the effects of three metabolites of verapamil (norverapamil, D617 and D620) on the protein binding of verapamil enantiomers and reported no clinically significant protein binding displacement interaction between verapamil and its metabolites. In their study they found that norverapamil concentrations up to 1000 ng/ml and D617 and D620 concentrations up to 5000 ng/ml caused no displacement of verapamil enantiomers from their protein binding sites. Only at norverapamil concentrations of 5000 ng/ml was a displacement interaction noted. As the highest norverapamil steady state concentrations observed in man are usually less than 500 ng/ml, standard dosing of verapamil would not be expected to produce high enough norverapamil concentrations to cause protein binding displacement. The protein binding of verapamil enantiomers was also investigated in maternal and fetal serum and found to be stereoselective [28]. In that study the free fraction of both enantiomers of verapamil was higher in fetal than maternal serum. They also measured the concentration of α_1-acid glycoprotein in maternal and fetal serum and found it approximately four times lower in fetal than maternal serum. The author

concluded that this difference in binding protein concentration is one of the factors responsible for the difference in binding between maternal and fetal serum. Recently, Abernathy *et al.* [21] reported stereospecific protein binding of verapamil enantiomers in young and elderly human and found that the binding of R-verapamil was higher than S-verapamil in both groups (94% vs 91%) and there was no difference in the young and elderly human. These findings are consistent with our results, however Abernathy *et al.* [21] did not study the binding of norverapamil enantiomers.

IR *versus* CR Administration

Using non-stereospecific assays, the bioavailability of CR verapamil was found to be either lower [40-41], similar [42], or higher [1] than the IR preparations at the same dosing level. Compared to non-stereospecific studies, there are few reports describing the enantioselective disposition of verapamil. These studies determined that the disposition of verapamil was stereoselective and that the main metabolite, norverapamil, was formed via first-pass hepatic metabolism [30-35]. In fact, the bioavailability of R-verapamil was reported as being 2 to 5 times greater than that of S-verapamil due to stereoselective first pass metabolism of S-verapamil after oral doses [21,28,35-36].

On comparing data published by several authors, it was hypothesized [42] that the extent of verapamil first pass metabolism was determined by Michaelis-Menten kinetics. It was further suggested that absolute bioavailability increased

when the drug exposed to the liver upon first pass exceeded the ability of the liver to extract the drug. In a clinical investigation [43] it was found that the concentration-effect curve of verapamil (concentration *versus* % change in PR interval) was shifted to the right after the administration of CR formulation (240 mg once daily) as compared to IR formulation (80 mg three times daily). These authors hypothesized that the verapamil concentrations obtained after administration of the 240 mg tablets represented an unknown mixture of isomers with differing dromotropic potency. It was suggested that the differences between the responses after administration of different preparations were related to the drug input rate; under conditions of a slower drug input rate, the liver's ability to extract drug may lead to an overall greater loss in S-verapamil during the first pass. Therefore, qualitatively different responses to verapamil oral preparations may be explained by differences in the S/R verapamil ratio in the plasma concentration-time curves. Mehvar [44] theorized that depending on the input rate of verapamil, the AUC S/R ratios differed as a function of hepatic first-pass metabolism which, in fact, was input rate-dependent. Abernethy [21] also reported a dramatic and significant reduction in the concentration of S-verapamil after CR administration compared with IR administration. The present study supported the findings of Abernethy [21] and others [44-45]. The enantiomer kinetics of the major active metabolite norverapamil, however was not studied in these previous reports.

Previous studies have reported that the site of stereoselective first-pass metabolism of verapamil is the liver [36-37], however metabolism in the gut wall

and by the gut flora cannot be ruled out unless specifically investigated. Although it is known that other drugs may be metabolized by gut wall [46,47], there is little known regarding the influence of this organ on the metabolism of verapamil in human. Interestingly, Koch found that verapamil is converted to norverapamil by intestinal flora in rats [48]. It may be hypothesized that after the IR administration, metabolism of verapamil may be saturable at any, or all levels, including the microflora, the gut wall or the liver. In any event, the specific metabolic events require further characterization and will be the topic of future studies.

As it is desirable to obtain consistent clinical effects (e.g., sustained blood pressure reduction), it may be more appropriate to define the peak-to-trough fluctuation in these subjects, rather than to merely report AUC values. It may be likely, therefore, that minimal eutomer concentration fluctuations would offer the most beneficial clinical effect(s). Investigation of this fluctuation revealed statistically significant differences between the IR and CR products. In fact, there was greater fluctuation after CR administration if the dosing intervals were compared, since due to the long dosing interval, the Cmin values were lower than the Cmin values after IR administration. On the contrary, if fluctuation per unit time was compared (%fluctuation per hour), there was no statistically significant difference between the two products.

The implication of these formulation-dependent pharmacokinetic findings may have significant impact on the clinical management of patients. It may be argued that R:S concentration ratios of verapamil and norverapamil may determine

overall clinical effect. Clinical effect, therefore, may be formulation-dependent. This latter point warrants further investigation.

CONCLUSION

In conclusion, our study determined that the disposition of verapamil and norverapamil enantiomers was stereoselective regardless of the formulation administered and that there was a trend of decreased systemic availability of the more active S-verapamil. The possible reasons that others [49-50] detected and we were unable to detect statistically significant differences between the products may include the sensitivity of the assay and very high intersubject variability. The higher intersubject variability in the present study may be due to the presence of both male and females subjects, since recent studies have shown gender-related differences in the stereoselective disposition of verapamil [20]. A trend towards higher systemic availability of S-verapamil after administration of IR formulation, compared to CR formulation suggests saturable pre-systemic metabolism. The precise mechanism of this pre-systemic metabolism may include hepatic, gut wall, or gut microflora factors, or a combination thereof. On comparing total (S plus R) verapamil after IR or CR administration no difference in the relative bioavailability of the two formulations was detected. The clinical relevance of these observations remains to be determined when comparing IR and CR formulations.

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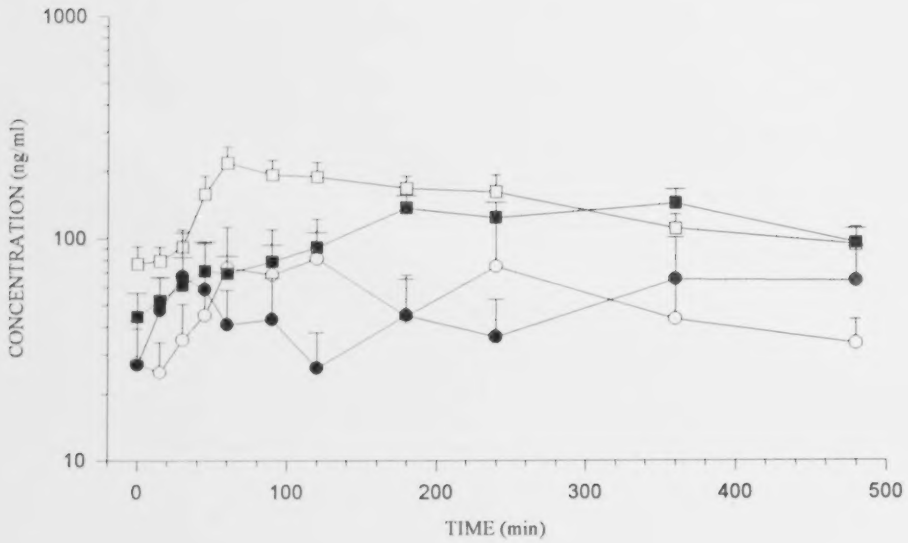

Figure 3-1. Mean plasma concentration versus time profile for enantiomers of verapamil after IR and CR administration. S-verapamil-IR = ○, R- verapamil-IR = □, S-verapamil-CR = ●, R- verapamil-CR = ■. Data are expressed as mean ± SEM.

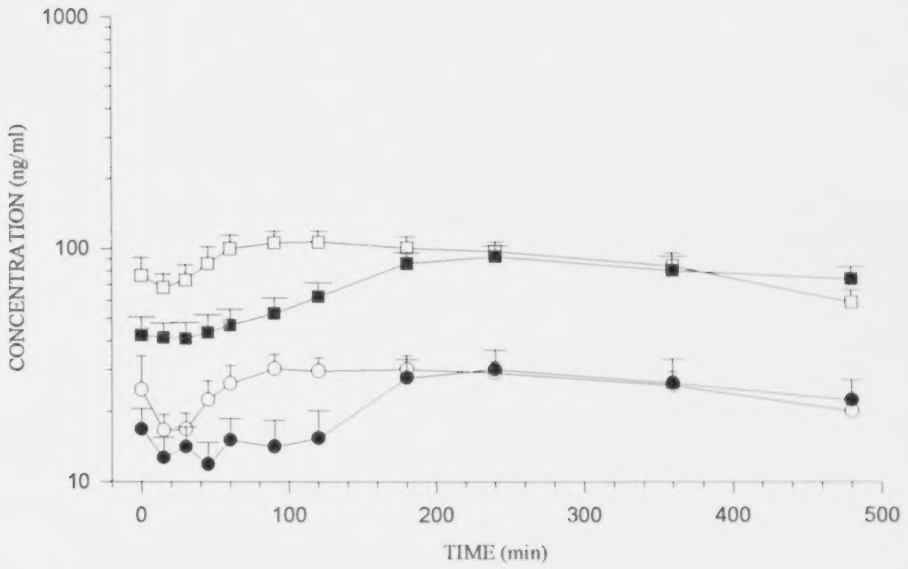

Figure 3-2. Mean plasma concentration versus time profile for enantiomers of norverapamil after IR and CR administration of verapamil. S-norverapamil-IR = ○, R- norverapamil -IR = □, S-norverapamil -CR = ●, R- norverapamil-CR = ■ Data are expressed as mean ± SEM

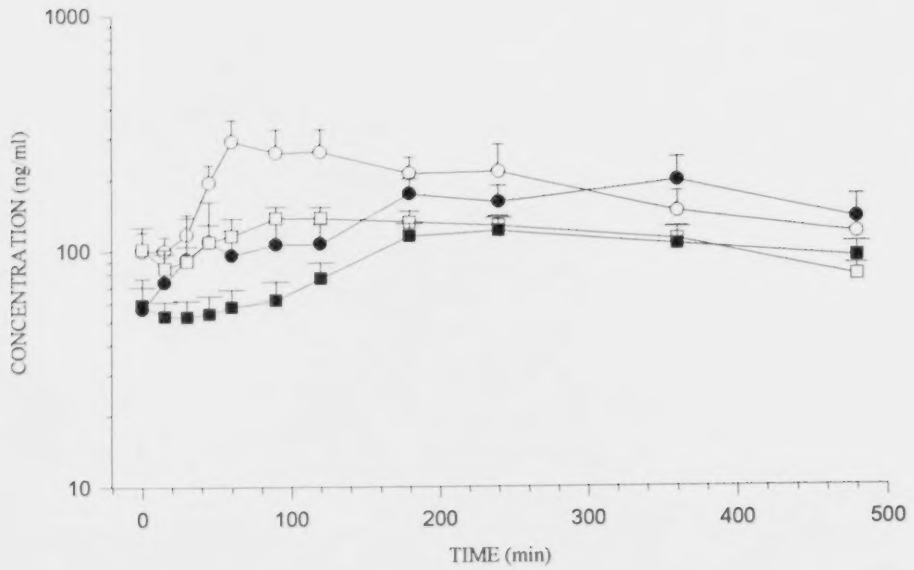

Figure 3-3. Mean plasma concentration versus time profile for total verapamil and total norverapamil after IR and CR administration. verapamil-IR = ○, verapamil-CR = ●, norverapamil-IR = □, norverapamil-CR = ■. Data are expressed as mean ± SEM.

Table 3-1. Characteristics for subjects. Data are expressed as mean ± SEM.

Age (years)	27 ± 3.1
Sex	4 Males 7 Females
Weight (Kg)	69 ± 4.1
Height (cm)	170 ± 3.0

Table. 3-2. Bioequivalence parameters of verapamil after administration of immediate release (IR) and controlled release (IR) verapamil. Data are expressed as mean ± SEM.

	IR	CR
S-Verapamil		
▾AUC (ng/ml)h	*1086 ± 619	*952 ± 497
Cmax (ng/ml)	*98.2 ± 40	*66.7±31.6
Cmin (ng/ml)	*18.4 ± 11	*11.6 ± 10
Tmax (h)	2.27 ± 0.7	7.23 ± 2.0
CL/F (L/h)	*1793 ± 255	*1324 ± 600
CL/F (L/h/Kg)	*8.34 ± 4.2	*19.1± 8.6
Fluctuation%	81.7 ± 6.3**	95.8 ± 3
Fluctuation% per h	3.4 ± 0.8	3.9 ± 0.1
R-Verapamil		
▾AUC (ng/ml)h	3389 ± 515	3082± 980
Cmax ng/ml	236 ± 29.6	241 ± 77.7
Cmin ng/ml	62.6 ± 12	33.6 ± 8.4
Tmax (h)	1.57 ± 0.2	6.9 ± 2.0
CL/F (L/h)	45.7 ± 7.4	70.8 ± 4.0
CL/F (L/h/Kg)	0.74 ± 0.1	0.95 ± 0.2
Fluctuation%	72.2 ± 4.3***	83.8 ± 3.0
Fluctuation% per h	3.0 ± 0.5	3.5 ± 0.1
Total (S + R) Verapamil		
▾AUC (ng/ml)h	4473 ± 1008	4034±1193
Cmax ng/ml	334 ± 63.7	308 ± 90.5
Cmin ng/ml	81.1 ± 17.8	45.2± 17.3
CL/F (L/h)	73.6 ± 10	113 ± 24
CL/F (L/h/Kg)	1.13 ± 0.2	1.56 ± 0.4
Fluctuation%	73.7 ± 4.4****	85.4 ± 3.1
Fluctuation% per h	3.1 ± 0.55	3.5 ± 0.1
R:S Ratio (AUC)	13.2 ± 6.4	19.2 ± 9.0

▾AUC 0-8 h x 3 for IR and 0-24 h for CR

* P < .01 S-verapamil *vs.* R-verapamil paired Student's *t* test

** P < .029 S-verapamil-IR *vs.* S-verapamil-CR paired Student's *t* test

*** P <.019 R-verapamil-IR *vs.* R-verapamil-CR paired Student's *t* test

**** P < .022 verapamil-IR *vs.* verapamil-CR paired Student's *t* test

Table. 3-3 Bioequivalence parameters of norverapamil after administration of immediate release (IR) and controlled release (CR) verapamil. Data are expressed as mean ± SEM.

	IR	CR
S-Norverapamil		
▾AUC (ng/ml)h	*608 ± 79	*448± 95.6
Cmax (ng/ml)	*34 ± 4.6	*34.2 ± 6.9
Tmax (h)	2.7 ± 1.5	5.02 ± 1.58
R-Norverapamil		
▾AUC (ng/ml)h	2143 ± 259	1693± 235
Cmax (ng/ml)	115 ± 12.8	100 ± 11.8
Tmax (h)	1.8 ± 0.4	6.8 ± 2
Total (S + R) Norverapamil		
▾AUC (ng/ml)h	2752 ± 311	2141±317
Cmax (ng/ml)	150 ± 16	135 ± 18
R:S Ratio (AUC)	3.7 ± 0.3	4.6 ± 0.5

▾AUC 0-8 h x 3 for IR and 0-24 h for CR

* P < .05 S-NORVERAPAMIL *vs.* R-NORVERAPAMIL paired student's *t* test

Table 3-4. Protein binding of verapamil enantiomers, with and without norverapamil, in young and elderly humans. Data are expressed as mean ± SD

	Protein Binding, (% unbound)			
	S-V	**R-V**	**S-V (with NV)**	**R-V (with NV)**
Young humans *n=4*	12.6±1.0[a]	4.0±0.8	11.6±1.9[a]	4.9±0.9
Elderly humans *n=4*	11.3±1.0[a]	5.5±1.7	10.7±1.7[a]	5.2±1.6

[a] Significantly different from corresponding antipode.
(V= verapamil, NV= norverapamil)

3-5. Protein binding of norverapamil enantiomers in young and elderly humans. Data are expressed as mean ±SD.

	Protein Binding, (% unbound)	
	S-NV	R-NV
Young humans		
n=4	7.5±0.5[a]	5.8±0.3
Elderly humans		
n=4	8.2±1.3[a]	6.1±1.3

[a] Significantly different from corresponding antipode.
(NV=norverapamil)

4. PHARMACOKINETICS OF THE ENANTIOMERS OF VERAPAMIL AFTER INTRAVENOUS AND ORAL ADMINISTRATION OF RACEMIC VERAPAMIL IN A RAT MODEL

INTRODUCTION

The calcium channel blocking drug, verapamil is an effective agent in the treatment of supraventricular tachyarrhythmias, angina pectoris and hypertension [1]. Verapamil is administered as a racemate; the two enantiomers differ considerably in their pharmacological potency with the S-enantiomer being 10-20 times more potent than the R-enantiomer in terms of negative dromotropic effect on atrioventricular conduction in man [2], in dog [3], and in rabbit [4]. The pharmacokinetics of verapamil enantiomers also differ from each other after intravenous (i.v.) and oral (p.o.) doses in both human [5] and animals [4,6-10]. Although the kinetics of the individual enantiomers of verapamil in human have been extensively studied, a review of the literature reveals that there is a scarcity of stereospecific studies of verapamil in animals [4, 10]. In view of the increasing interest in interspecies scaling-up of absorption [11-12] and disposition kinetics

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[13-15] and of the findings that the rat may serve as a good model for the human in p.o. absorption [10], it was decided to study, in detail, the kinetics of verapamil enantiomers in male Sprague-Dawley rats.

Studies conducted on the kinetics of racemic verapamil in rats [6, 8-9], measuring racemic verapamil indicated that, similar to man, verapamil undergoes extensive hepatic metabolism with a high hepatic extraction ratio. Additionally, studies using the rat liver microsomal fraction [16] or isolated perfused rat liver [17] demonstrated stereoselectivity in different metabolic pathways of verapamil. However, the kinetics of the individual enantiomers of verapamil, after the administration of racemic verapamil in rats are unknown. The major objective of the present investigation was to evaluate the kinetics of the verapamil enantiomers following i.v. and p.o. administration in the male Sprague-Dawley rat. These data will be used to contrast the disposition of verapamil to that observed in humans.

MATERIALS AND METHODS

Chemicals

Racemic verapamil hydrochloride was purchased from Sigma Chemical Co (St Louis, MO, USA). Internal standard, (+)-naproxen chloride, (I.S.) was synthesized in our laboratory by reacting thionyl chloride with (+)-naproxen and purified by repeated recrystallization. Thionyl chloride and (+)-naproxen were

obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). Verapamil hydrochloride solutions (1.0 mg ml^{-1}) for intravenous administration and 10 mg ml^{-1} for oral administration) were prepared by dissolving the drug in normal saline. Ultrafiltration units (Amicon Inc., Beverly, MA, USA) with a 1 ml sample cup and molecular weight cutoff of 30K were used for protein binding study. All other chemicals and reagents were HPLC or analytical grade.

Surgery and Animal Maintenance

Male Sprague-Dawley rats weighing between approximately 200 and 300 g were used for the study. A total of 12 rats were catheterized with silastic tubing (0.025" i.d. X 0.037" o.d.; Dow Corning, Midland, MI, U.S.A.) at the right jugular vein. Immediately prior to surgery, rats were anesthetized via intraperitoneal sodium pentobarbital (MTC Pharmaceutical Cambridge, Ontario, Canada). The animals were allowed to recover overnight prior to the experiment and were individually stored in 18" X 9.5" X 8" polycarbonate rodent cages. The animals were fasted at least 12 hours before the administration of verapamil dose.

Drug Administration and Sample Collection

A group of six rats received racemic verapamil i.v. and the other group of six rats received racemic verapamil p.o. dosage. Since the R- and S-verapamil were individually detected, this was a parallel group design study. Racemic verapamil dissolved in normal saline was administered, 0.5 mg kg^{-1} of each

enantiomer *via* the jugular vein cannula in the case of i.v. administration and 5 mg kg^{-1} of each enantiomer *via* feeding tube in the case of p.o. administration. After administration of the verapamil dose, the cannula or the feeding tube was flushed with approximately 1 ml of normal saline. Blood (0.25 ml) was collected from the jugular vein cannula just prior to, and at 0.033, 0.166, 0.33, 0.5, 0.75, 1, 1.5, 2, 3 and 5 h after drug administration. Between each blood sample collection 0.2 ml normal (0.9%) saline was administered *via* the jugular vein cannula as fluid replacement, and the cannula was heparinized (10 U/ml). Blood samples were centrifuged and the plasma portion was separated and immediately frozen at -20° C until analyzed. Animals were given water *ad libitum* throughout the study and food was withheld only during the two h period immediately following drug administration.

Stereospecific HPLC Analysis of Verapamil

Concentrations of S- and R-verapamil in plasma were determined utilizing a stereospecific high-performance liquid chromatographic (HPLC) method developed in our laboratory [18]. The coefficient of variation of the assay for control plasma standards was 1.5% to 9.0%. The lower limit of sensitivity was 5 ng ml^{-1} of each enantiomer.

Protein Binding Study

Rat blood samples were collected from four young (8 weeks old) and four elderly (60 weeks old) male Sprague-Dawley rats. The protein binding of verapamil enantiomers was investigated in the rat plasma by using the same method described in the previous chapter.

Pharmacokinetic Data Analysis

The area under the plasma concentration-time curve ($AUC_{0-\infty}$) was calculated by the trapezoidal rule using a Lagran computer software program [20]. The area from the last concentration point (C_{last}) to infinity was calculated as C_{last}/λ_n, where λ_n was the terminal elimination rate constant, calculated by least square regression line with data points in the terminal elimination phase. Systemic clearance (Cl_s) was calculated as $D_{i.v.}/AUC_{0-\infty}$, where $D_{i.v.}$ was the enantiomeric dose administered i.v. and $AUC_{0-\infty}$ was the corresponding area under the plasma enantiomer concentration-time curve. Similarly, oral clearance (Cl_o) was calculated as $D_{oral}/AUC_{0-\infty}$, where D_{oral} was the enantiomeric dose administered orally and $AUC_{0-\infty}$ was the corresponding area under the plasma enantiomer concentration-time curve. The apparent volume of distribution (Vd_β) was calculated by dividing corresponding Cl_s by λ_n. The absolute bioavailability (F) was calculated as $(Dose_{i.v.} \times AUC_{oral})/ (Dose_{oral} \times AUC_{i.v.})$ and extraction ratio (E) was calculated by subtracting F from 1.

Statistical Analysis

Comparisons between the S- and R-verapamil concentrations observed in rats administered the racemate were assessed utilizing a Student's *t* test for paired data. Differences in the protein binding of verapamil enantiomers observed in young and elderly rats were performed using two way analysis of variance (ANOVA). All *t* tests were two-tailed, with the level of significance pre-set an $\alpha = 0.05$. Results are expressed as mean ± SD.

RESULTS

Intravenous Pharmacokinetics

The mean plasma concentration-time profiles of R- and S-verapamil after i.v. administration of racemic verapamil are shown in figure 4-1. The drug data were fitted to the biexponential equation using Sigma Plot computer program which indicated that the plasma concentration declined in a biphasic fashion. At almost every time point the plasma concentration of R-verapamil was approximately 40-50% that of S-verapamil. The i.v. pharmacokinetic data derived from figure 4-1 is summarized in table 4-1. The systemic clearance of R-verapamil was on average 48% greater than that of S-verapamil (40.9 vs. 27.7 ml $min^{-1}kg$). The apparent volume of distribution of R-verapamil was also significantly greater than that of S-verapamil (5.30 vs. 3.34 l kg^{-1}). The higher Cl_s and Vd_β of R-

verapamil had a counteracting effect on the elimination half-life such that there was no difference between the enantiomers in these pharmacokinetic parameters.

Oral Pharmacokinetics

The mean plasma concentration-time profiles of R- and S-verapamil after p.o. administration of racemic verapamil are shown in figure 4-1b and the corresponding pharmacokinetic parameters are summarized in table 4-2. As is apparent from figure 4-1b, the oral clearance of R-verapamil was of the order of 2.5 times that of S-verapamil indicating that presystemic elimination of racemic verapamil is selective for the R-enantiomer. As was seen in the i.v. data, the elimination half-life of the two enantiomers were similar being around 1.7-2.3 h. The oral bioavailability of R-verapamil was 4.1%, while that of S-verapamil was 7.4%. The extraction ratio of R-verapamil was significantly greater than that of S-verapamil.

Protein Binding

The extent of binding of verapamil enantiomers to plasma proteins in different study groups is summarized in Tables 4-3. The binding of S-enantiomers was greater than R-enantiomers in young and elderly rats. There were no significant difference between age groups. At the concentrations studied, norverapamil did not alter the binding of verapamil enantiomers (Table 4-3).

DISCUSSION

In this study, the pharmacokinetics and bioavailability of the enantiomers of verapamil were studied in the rat after single i.v. and p.o. dosing of racemic verapamil. The method used to quantitate the plasma concentration of the individual enantiomers of verapamil involved a stereospecific HPLC assay using a single chiral column.

Both the systemic and oral clearances of R-verapamil were significantly higher than that of S-verapamil in the rat. These findings are in contrast to the reports of the i.v. [21] and p.o. [22] kinetics of the verapamil enantiomers in man. After intravenous administration of a pseudoracemic mixture (50% dideuterated R-verapamil:50% unlabeled S-verapamil) in man, Eichelbaum *et al.* [21] found the systemic plasma clearance of S-verapamil was 80% greater than that of R-verapamil. In contrast, in the present study using a rat model, the systemic clearance of the R-verapamil was 48% greater than that of S-verapamil. As the hepatic blood flow was the same for both enantiomers, the higher systemic clearance for the R-verapamil reflects a higher extraction ratio. This was confirmed by the results of the p.o. dosing experiments, where the oral clearance, which reflects the intrinsic ability of the liver to clear the drug, of R-verapamil was over 2.5 times that of S-verapamil. Needless to say, the determination of hepatic intrinsic clearance in this manner assumes complete absorption from the gut. This was further reflected in the approximately 2-fold oral bioavailability of S-verapamil over that of R-verapamil (7.5 vs. 4.1%). Man also shows a stereoselective

presystemic uptake of verapamil [21-22]. However, in man the S-enantiomer presystemic uptake is greater (4 to 5-fold) than that of the R-enantiomer. The oral bioavailability (S-verapamil 0.074 ± 0.031 and R-verapamil 0.041 ± 0.011) observed in the present study is comparable with hepatic availability values (S-verapamil 0.069 ± 0.030 and R-verapamil 0.046 ± 0.025) reported by Mehvar *et al.* [17] in an isolated perfused rat liver study. These findings suggest that the liver is most likely responsible for stereoselective presystemic uptake of verapamil enantiomers in the rat. Although, in an *in vitro* study, Koch *et al.* [23] found that verapamil is also metabolized by intestinal flora in the rat cecal contents, it is somewhat doubtful that a significant portion of the dose reaches the colon after oral administration of verapamil solution.

In an *in vitro* study, Nelson *et al.* [16] compared the verapamil metabolism of human and rat using liver microsomal fractions from both species. In their study they found considerable similarities in the metabolic pathways of the two species and concluded that a similar set of cytochrome P-450 isozymes may be responsible for this biotransformation. They also suggested that the different stereoselectivities observed by the two species may be due to the structural differences in these enzymes and/or due to free fraction of the drug.

In addition to the stereoselective clearances, the apparent volume of distribution for the enantiomers of verapamil exhibited stereoselectivity in the rat. The volume of distribution values of R-verapamil were approximately 58% greater than those of S-verapamil. In man, the opposite situation occurs both qualitatively

and quantitatively [24]. The physiological relationship that relates a drug's apparent volume of distribution to physiological volumes, the binding in plasma, and the binding in tissues, first proposed by Gillette [25] is

$$V_d = V_p + V_t (f_p / f_t)$$

where V_p is the plasma volume (approx. 5% body weight), V_t is the tissue volume (approx. 55% body weight), f_p and f_t are the free fraction in the plasma and tissues, respectively. In the protein binding experiment we found higher f_p value of R-verapamil than S-verapamil in rat. Simmilar results were also reported by others [26-27]. Based on this relationship, it is possible to speculate that the larger apparent volume of distribution of R-verapamil can be accounted for by its higher free fraction in plasma. Interestingly, the same rationale can be used to explain the higher volume of distribution for S-verapamil in human [27] where the f_p of S-verapamil is higher than R-verapamil.

The pharmacokinetics of enantiomers of verapamil have also been studied in dog and rabbits [4, 10]. Bai *et al* [10] reported stereoselective disposition of verapamil in dog and found that the Cl_s, Cl_o and Vd_β values of S-verapamil were greater than that of R-verapamil and the oral bioavailability of R-verapamil was 14-fold greater than that of S-verapamil. These differences between the bioavailabilities of the enantiomers in dog were much greater than those found (4-fold) in man. Contrary to human, dog and rat, Giacomoni *et al.* [4] reported that the pharmacokinetics of verapamil enantiomers were not stereoselective in rabbits. In that study they also determined that there was no difference in the protein

binding of the enantiomers of verapamil. Recently Mehvar *et al.* [28] studied the influence of protein binding on the direction of stereoselectivity in the kinetics of verapamil in isolated perfused rat livers using bovine and human albumin. They found that the direction of stereoselectivity in the first pass metabolism was mainly governed by the stereoselectivity in the protein binding of the drug.

In conclusion, this study found that, similar to human, verapamil is stereoselectively metabolized by rat after p.o. and i.v. administration and the apparent oral bioavailabilities of both enantiomers are low. Stereoselectivity in the systemic and presystemic clearance in the rat is in the opposite direction as in the human. The extent of stereoselectivity after p.o. administration was much higher than after i.v. administration which reflected differences in the intrinsic clearances of verapamil enantiomers.

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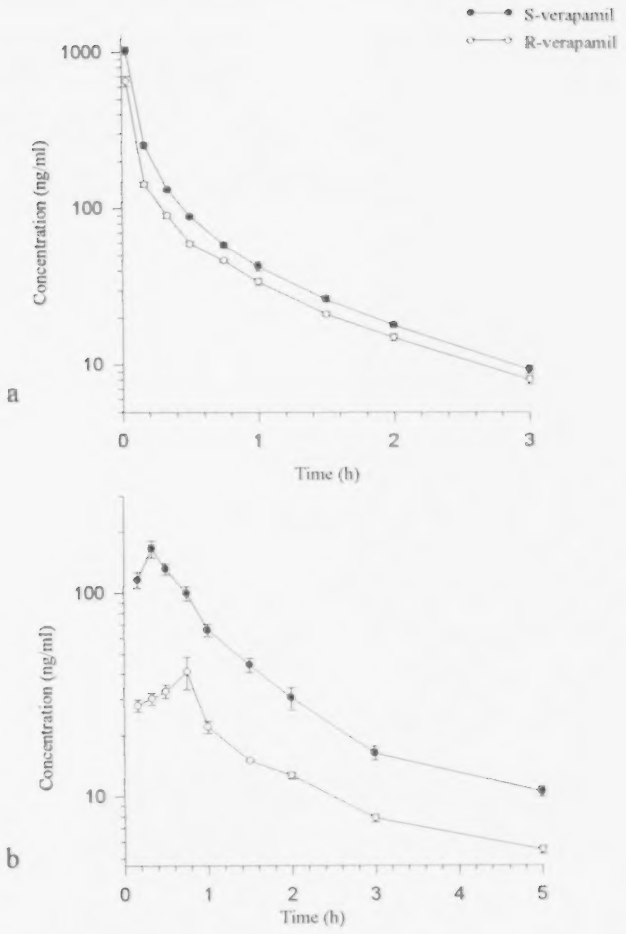

Figure 4-1

(a). Plasma concentration *versus* time profile for enantiomers of verapamil after intravenous administration of racemic verapamil; R-verapamil = ○, S-verapamil = ●. Each point is the group mean ± S.D. (n=6, no drug was detected at 5 h time point).

(b). Plasma concentration *versus* time profile for enantiomers of verapamil after oral administration of racemic verapamil; R-verapamil = ○, S-verapamil = ●. Each point is the group mean ± S.D. (n=6).

Table. 4-1. Pharmacokinetic parameters of verapamil enantiomers after intravenous administration of racemic verapamil in rats. Data are expressed as mean ± SD.

	Weight	$AUC_{0-\infty}$ ($\mu g\ h\ l^{-1}$)		CL_s ($ml\ min^{-1}\ kg^{-1}$)		$t_{1/2}$ (h)		V_{dss} ($l\ kg^{-1}$)	
RAT	(g)	S	R	S	R	S	R	S	R
1	210	300	198	23.3	35.3	0.53	0.85	1.27	3.09
2	210	260	157	26.8	44.4	0.63	0.64	1.74	2.93
3	215	267	194	26.7	36.9	0.82	0.94	2.15	3.49
4	210	284	184	24.6	38.0	2.01	1.78	5.10	6.99
5	220	303	229	24.2	31.9	2.32	2.82	5.52	8.89
6	220	439	314	16.7	23.3	2.57	2.76	4.24	6.36
Mean	214	309[a]	212	23.7[a]	34.9	1.48	1.63	3.34[a]	5.30
SD	4.9	65.8	54.8	3.7	7.0	0.91	0.97	1.83	2.47

[a] Significantly different from corresponding antipode.

Table. 4-2. Pharmacokinetic parameters of verapamil enantiomers after oral administration of racemic verapamil in rats. Data are expressed as mean ± SD.

	Weight	$AUC_{0-\infty}$ ($\mu g\,h\,l^{-1}$)		CL_{oral} ($ml\,min^{-1}\,kg^{-1}$)		$t_{1/2}$ (h)		Cmax (ng/ml)		Tmax (ng/ml)		F	
RAT	(g)	S	R	S	R	S	R	S	R	S	R	S	R-----
1	215	204	90	352	792	0.95	2.29	212	47.2	20	20	0.066	0.044
2	215	359	79	200	907	1.31	1.95	286	42.2	20	20	0.134	0.048
3	215	213	51	328	1400	1.55	1.23	218	30.9	20	20	0.081	0.026
4	210	133	104	527	673	2.25	3.71	74	30.6	20	45	0.046	0.056
5	220	185	73	396	998	2.56	2.45	69	30.2	30	30	0.060	0.032
6	210	232	123	301	566	1.31	2.74	192	34.5	10	45	0.055	0.041
Mean	214	221[a]	87	351[a]	889	1.65	2.39	175[a]	35.9	20	30	0.074[a]	0.041
SD	3.7	75	25	109	294	0.62	0.82	86	7.1	6.3	12	0.031	0.011

[a] Significantly different from corresponding antipode

Table 4-3. Protein binding of verapamil enantiomers, with and without norverapamil, in young and elderly rats. Data are expressed as mean ± SD.

Protein Binding, (% unbound)

	S-V	R-V	S-V (with NV)	R-V (with NV)
Young Rats n=4, pooled	5.1±1.6[a]	10.9±1.1	5.9±1.2[a]	9.8±1.0
Elderly Rats n=4, pooled	4.7±0.3[a]	9.1±2.7	5.2±1.2[a]	10.6±1.2

[a] Significantly different from corresponding antipode.
(V= verapamil, NV= norverapamil)

5. THE INFLUENCE OF METOPROLOL ON THE PHARMACOKINETICS OF THE ENANTIOMERS OF VERAPAMIL IN A RAT MODEL

INTRODUCTION

Verapamil is an inhibitor of the slow inward current of calcium in a variety of cardiac tissues. The drug is widely used in therapy of hypotension, supraventricular arrhythmias and angina pectoris [1]. Although verapamil is administered as a racemate, most of its cardiovascular effects are mediated predominantly by the S-enantiomer [2]. In order to relate drug concentration to effect it is important to understand the biological fate of the enantiomers of verapamil, especially that of S-verapamil. This is relevant for verapamil since its oral and systemic clearances as well as its distribution and plasma protein binding are stereoselective both in humans [3-6] and in rats [7-9]. Although numerous studies have been reported on drug interactions with racemic verapamil [10], little is known about the effects that other drugs may have on the disposition of the individual enantiomers.

Verapamil acts mainly by inhibiting myotonic tone of veins and arteries,

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and thereby diminishes both ventricular preload and afterload [11]. It also has a well-documented direct inhibitory effect on coronary vessel tone [11]. These properties are not characteristic of β-adrenoreceptor blocking drugs and provide a rationale for the combination of verapamil and β-adrenoreceptor blocking drugs. This combination provides synergistic efficacy in patients with ischemic heart disease and chronic stable angina, who remain symptomatic with either agent alone [12]. The potential for cardiac depression and circulatory collapse when verapamil is given in combination with β-adrenoreceptor blockers is particularly noteworthy [13]. Both clinical reports [14-16] and animal studies [17-18] have suggested that the combination of calcium channel blockers and β-adrenoreceptor blockers may decrease ventricular pump performance. Although pharmacodynamic interactions were assumed to be the cause of these adverse reactions, other studies suggested that the combination of β-adrenoreceptor blockers with calcium channel blockers may result in pharmacokinetic interactions [10]. The possible effect of metoprolol on the pharmacokinetics of verapamil enantiomers have been investigated.

EXPERIMENTAL

Materials

Racemic verapamil hydrochloride and racemic metoprolol tartrate were purchased from Sigma Chemical Co (St Louis, MO, USA). Internal standard, (+)-

naproxen chloride, (I.S.) was synthesized in our laboratory by reacting thionyl chloride with (+)-naproxen and purified by repeated recrystallization. Thionyl chloride and (+)-naproxen were obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). Verapamil hydrochloride solutions (1.0 mg ml^{-1} for intravenous administration and 10 mg ml^{-1} for oral administration) and metoprolol tartrate solutions (3.0 mg ml^{-1} for intravenous administration and 20 mg ml^{-1} for oral administration) were prepared by dissolving the drug in normal saline. All other chemicals and reagents were HPLC or analytical grade.

Pharmacokinetics Studies

Healthy male Sprague-Dawley rats weighing between approximately 200 and 300 g were used for the study. Each rat was catheterized with silastic tubing (0.025" i.d. X 0.037" o.d.; Dow Corning, Midland, MI, U.S.A.) at the right jugular vein according to the method described earlier [9]. The animals were allowed to recover overnight prior to the experiment and were individually stored in 18" X 9.5" X 8" polycarbonate rodent cages. The animals were deprived of food but given free access to water for about 12 hours prior to the administration of drugs.

Drugs were administered to the conscious rats. In the oral administration study, each rat (n=6) received a single dose of racemic verapamil (10 mg kg^{-1}) in combination with racemic metoprolol (20 mg kg^{-1}) dissolved in normal saline via a feeding tube. In the intravenous study, each rat (n=6) received a single dose of

racemic verapamil (1 mg kg^{-1}) in combination with metoprolol (3 mg kg^{-1}) via the jugular vein cannula. After administration of the dose, the cannula or the feeding tube was flushed with approximately 1.0 ml of normal saline. Each rat was kept in an individual cage without restraint. Blood (0.25 ml) was collected from the jugular vein cannula just prior to, and at 0.033, 0.166, 0.33, 0.5, 0.75, 1, 1.5, 2, 3 and 5 h after drug administration. Between each blood sample collection 0.2 ml normal (0.9%) saline was administered *via* the jugular vein cannula as fluid replacement; the cannula was heparinized (10 U/ml). Plasma was separated immediately by centrifugation and stored at -20° C until assayed. Animals were given water *ad libitum* throughout the study and food was withheld only during the two h period immediately following drug administration.

The results of the present study will be compared with the results of our previous study [9] done two weeks earlier in a similar fashion, except metoprolol was not administered with verapamil.

Liver Microsome Studies

Healthy male Sprague-Dawley rats (200-300 g) were used in these *in vitro* studies. Freshly excised rat livers were rapidly placed in ice-cold 1 mM phosphate-sucrose buffer (pH 7.4). The organs were homogenized by a Potter Elvehjem tissue grinder (Talboys Engineering, Montrose, PA). The homogenate was centrifuged for 20 min at 9,000 *g* at 4 ^{0}C, to yield the supernatant fraction. The supernatant was then centrifuged at 100,000 *g* for 60 min at 4 ^{0}C. The microsomal

pellets obtained were suspended in buffer and recentrifuged at 100,00 *g* for an additional 60 min. The final pellet was resuspended in phosphate-sucrose buffer. Protein concentration was determined by the Lowry method (Sigma Chemical Co. St Louis, MO, USA). The incubation reaction mixture was similar to that used by Hamann *et al.* [19] for *in vitro* hepatic metabolism of verapamil. Reaction mixture (total volume 3 ml) containing glucose-6-phosphate (20 mM), nicotinamide (40 mM), nicotinamide adenine dinucleotide phosphate (0.2 mM) (Sigma Chemical Co. St Louis, MO, USA)., $MgCl_2$ (60 mM) and 15 μg liver microsome in phosphate buffer (pH 7.4). In the control group (n=5), the mixture was spiked with 2 mg/l racemic verapamil. In the treatment group (n=5), the mixture was spiked with racemic metoprolol 2 mg/l, 30 min prior to the addition of 2 mg/l racemic verapamil. The mixture was shaken in a water bath at 37 ^{0}C. At 0, 0.5, 1, and 2 h after incubation, the reaction was stopped by transferring aliquots (0.5 ml) to tubes containing 0.5 ml 1 N NaOH. Samples were stored at -20 ^{0}C until assayed for verapamil enantiomers. Microsomal metabolism of verapamil in the treatment group was compared to that of control groups by measuring the % loss of verapamil versus time in the microsomal mixture.

Protein Binding Study

The effect of metoprolol on the protein binding of verapamil enantiomers was investigated in the rat plasma by using the same method described in the previous chapter.

Stereospecific HPLC Analysis of Verapamil

Concentrations of S- and R-verapamil in plasma were determined utilizing a stereospecific high-performance liquid chromatographic (HPLC) method developed in our laboratory [20]. The concentrations of verapamil enantiomers in the rat liver microsomes was determined by the same method. Since NaOH was already added to stop the reaction, the base was not used in the extraction step.

Pharmacokinetic Data Analysis

The terminal elimination half-life ($t_{1/2}$) for each enantiomer was determined by 0.693/β, where β (elimination rate constant) was calculated using the regression slope of the log-linear terminal elimination phase. The area under the plasma concentration-time curve ($AUC_{0-\infty}$) was calculated by the trapezoidal rule using a Lagran computer software program. Systemic clearance (Cl_s) was calculated by dividing the administered i.v. dose by the corresponding $AUC_{0-\infty}$. Similarly, oral clearance (Cl_o) was calculated by dividing the administered oral dose by the corresponding $AUC_{0-\infty}$. The volume of distribution (Vd_β) was calculated by dividing the Cl_s by β.

Statistical Analysis

Comparisons between the S- and R-verapamil concentrations observed in rats administered the racemate were assessed utilizing a Student's *t* test for paired data whereas enantiomeric concentrations of control and treatment groups were compared by independent Student's *t* test. All *t* tests were two-tailed, with the level of significance pre-set a $\alpha = 0.05$. Results are expressed as mean ± SD.

RESULTS

Pharmacokinetic study

The mean plasma concentration *versus* time curves, for S- and R-verapamil after oral administration of racemic verapamil with and without metoprolol are presented in figure 5-2 and pharmacokinetic parameters derived from these are listed in table 5-1. As expected, the pharmacokinetics of verapamil was stereoselective after the administration of racemic verapamil in the presence of metoprolol. The AUC and Cmax of R-and S-verapamil were significantly higher when the two drugs were given together than when verapamil was given alone. There was higher variability in the AUC values after oral than i.v. administration which reflected the variability in the presystemic metabolism. The time required to reach the peak was not significantly different between verapamil alone and

verapamil with metoprolol. The Cl_{oral} of both S- and R- verapamil decreased significantly during metoprolol co-administration.

The mean plasma concentration-time profile of R- and S-verapamil after i.v. administration of racemic verapamil with and without metoprolol are shown in figure 5-1. In the co-administration of verapamil and metoprolol, the plasma concentration of R- and S-verapamil declined more slowly than in the case of verapamil alone. The drug data were fitted to the biexponential equation using Sigma Plot computer program and the solid lines in figure 5-1 represent computer-fitted biexponential curves (data was also tried to fit in triexponentioal equation but biexponential gave the best fitted line). The corresponding pharmacokinetic parameters of R- and S-verapamil are summarized in table 5-2. The AUC values were significantly higher and Cl_s values were significantly lower for the combination than for verapamil alone. The systemic availability of verapamil enantiomers (AUC oral/ AUC i.v.) were higher when racemic verapamil was given with metoprolol than when it was given alone (R-verapamil:34.7 ± 39.6 *vs.* 4.1 ± 3.1%;S-verapamil:36.5 ± 39.6 *vs.* 7.4 ± 3.1%).

In Vitro Metabolism

The time-courses of *in vitro* metabolism of R- and S- verapamil are shown in figure 5-3. Even though the loss of verapamil was generally slower in treatment as compared to controls, statistically significant differences were only found at the two last data points. This was probably due to the reason that the interaction was studied only in one concentration of metoprolol (2 mg/l) and there might be more

pronounced differences if the study could have done at different higher concentrations. This effect was stereoselective for both control and treatment groups (S:R concentration ratio control:1.13 ± 0.02; treatment: 1.13 ± 0.01). There was a significantly greater percentage of the S-enantiomer remaining at each sampling time than R-verapamil in both groups.

Protein Binding

The extent of binding of verapamil enantiomers to plasma proteins with and without metoprolol is summarized in Tables 5-3. The binding of S-enantiomers was greater than R-enantiomers in both groups. At the concentrations studied, metoprolol did not alter the binding of verapamil enantiomers.

DISCUSSION

Drug interactions are a constant concern for the clinicians treating patients on multiple drug regimens. These may occur as "pharmacokinetic drug interactions" in which the amount of drug at the receptor site is altered, or as "pharmacodynamic drug interactions" which generally involve a modification of drug response by other mechanisms [21]. There have been a number of studies done in an effort to clarify the role of combination therapy with calcium channel blockers and β-blockers [22-23]. Nevertheless, this approach seems attractive since the calcium channel blockers increase the supply of oxygen to the ischemic myocardium, while β-blockers decrease demand. Unfortunately, adverse

reactions, including hypotension, bradycardia and cardiac failure are observed in a significant number of patients receiving combination therapy [14-16]. Pharmacodynamic interactions were assumed to be the cause of these adverse reactions, because of the additive cardiodepressant effects of combination therapy. Recently, attention has been drawn to the fact that pharmacokinetic interaction may be the cause of these adverse effects [24].

The idea of stereoselectivity in the pharmacokinetics and pharmacodynamics of chiral drugs have drawn considerable attention over the past decade [25]. Verapamil is a chiral drug and its enantiomers differ considerably in terms of pharmacological activity. Furthermore, its metabolism exhibits stereoselectivity in humans [3] and in rats [9]. The changes in the metabolism of verapamil, which alter the ratio of eutomer to distomer, can lead to different drug response although the total concentration remains unchanged. Echizen *et al.* [26] found that 3 to 4 times higher total plasma concentration of verapamil is required to produce the same negative dromotropic effect after oral, as after i.v. administration. This difference was explained by stereoselective first-pass metabolism which leads to preferential biotransformation of the more potent S-enantiomer, thus leading to a predominance of R-verapamil in plasma. Another way of modulating metabolic patterns is partial inhibition of metabolic pathways by other drugs. Such drug interactions may also influence stereoselective disposition.

In this study we investigated the effect of metoprolol administration on the stereoselective pharmacokinetics of the enantiomers of verapamil in the rat after

single i.v. and oral dosing of racemic verapamil. The plasma concentration of verapamil enantiomers after oral administration was significantly higher given metoprolol along with verapamil. Several mechanisms may account for this effect of metoprolol on plasma concentration of verapamil. One is an increase in the gastrointestinal absorption of verapamil. However, it has already been shown that verapamil is completely absorbed from the gastrointestinal tract [27]. A more subtle mechanism may be related to the fact that the presystemic metabolism of verapamil is capacity-limited [8]. It is also conceivable that metoprolol could cause verapamil to be absorbed more rapidly, which would lead to a higher concentration of verapamil in systemic circulation due to the capacity-limited presystemic metabolism. However, the fact that the time required to reach the peak plasma drug concentration (Tmax) was not affected by metoprolol suggests that there was no acceleration of verapamil absorption by metoprolol.

In the i.v. experiment, we found that metoprolol significantly decreased the Cl_s of verapamil enantiomers. Owing to the high extraction ratio for verapamil enantiomers, their Cl_s is predominantly dependent on the hepatic blood flow rate [8]. Changes in their Cl_s, therefore, may be a reflection of changes in the hepatic blood flow. The β-blockers competitively inhibit catecholamine neurotransmitters throughout the body at both cardiac receptors (β_1) and noncardiac receptors (β_2) [28]. Cardiac effects include a reduction in heart rate, cardiac output and cardiac work. These changes in the heart rate and cardiac output can alter the hepatic blood flow. Bailey *et al.* [29] reported a significant reduction in the heart rate of

humans after the administration of metoprolol. They also found that the combination of verapamil and metoprolol produced greater reduction in heart rate than did either agent alone. A recent study done in our laboratory by another investigator [30] also found a 65% reduction in cardiac output and 40 % reduction in the hepatic blood flow in male Sprague-Dawley rats after the administration of metoprolol. On the basis of assumptions of the venous equilibrium model [31-32], and because verapamil is completely eliminated by hepatic metabolism [8], the observed decrease in the Cl_s of verapamil enantiomers, may be due to a decrease in the hepatic blood flow. This decrease in Cl_s resulted in lengthening of the terminal $t_{1/2}$ of verapamil enantiomers without changing their volume of distribution. This was expected for a highly extracted drug like verapamil whose $t_{1/2}$ is dependent upon hepatic blood flow.

Comparing the oral to i. v. data, we found that metoprolol increased approximately 5 to 7 folds the average systemic availability of S-verapamil and R-verapamil. Based on the venous equilibrium model for hepatic clearance, a decrease in hepatic blood flow should not have increased the AUC after oral administration [32]. This increase in the systemic availability of verapamil enantiomers may reflect a decrease in hepatic presystemic clearance, i.e. decreased removal by the liver during the initial transit from the intestine to the systemic circulation. The increased AUC of verapamil enantiomers after oral administration of racemic verapamil reflects the decreased intrinsic clearance of verapamil enantiomers by metoprolol. This was evident by the observed decreased Cl_o of

verapamil enantiomers. To further investigate this effect, we studied the effect of metoprolol on the *in vitro* metabolism of verapamil enantiomers in the rat liver microsomal preparation. As shown in figure 5-3, the loss of verapamil enantiomers was significantly slower in the presence of metoprolol, as compared to verapamil alone. These results suggest that metoprolol increases the systemic availability of verapamil enantiomers by inhibiting presystemic elimination of verapamil in the liver.

The effects of other known inducers and inhibitors of hepatic microsomal enzymes, on the systemic availability of oral verapamil has also been reported by others [34-36]. Barbarash *et al.* [34] reported near total reduction in verapamil systemic availability by rifampin (a potent inducers of hepatic microsomal enzymes) in humans. On the other hand, co-administration of cimetidine (a known inhibitor of hepatic microsomal enzymes) significantly increased the systemic availability of verapamil enantiomers in humans [35] and in dog [36].

In conclusion, we demonstrated a pharmacokinetic interaction between verapamil and metoprolol in male Sprague-Dawley rats. Metoprolol alters both the i.v. and oral kinetics of verapamil by apparently two autonomous mechanisms, i.e. decrease in the hepatic blood flow and inhibition of the presystemic metabolism. The possible explanation, that decrease in the Cl_o was much more pronounced than Cl_s may include; 1) decrease in the intrinsic clearance and 2) saturation of the presystemic metabolism after oral administration.

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Table 5-1. Pharmacokinetic parameters of verapamil enantiomers after oral intravenous administration of racemic verapamil with and without metoprolol in rats. Data are expressed as mean ± SD.

	Without Metoprolol	With Metoprolol
S-Verapamil		
$AUC_{0-\infty}$ (μg h l^{-1})	221 ± 75[a b]	1457 ± 1074[b]
Cl_{oral} (ml min^{-1} kg^{-1})	350 ± 109[a b]	91 ± 59[b]
Cmax (ng ml^{-1})	172 ± 86[a b]	1134 ± 887[b]
Tmax (min)	20 ± 4	22 ± 4
$t_{1/2}$ (h)	1.6±0.6[a]	2.3± 1.1
R-Verapamil		
$AUC_{0-\infty}$ (μg h l^{-1})	87 ± 25[a]	821 ±831
Cl_{oral} (ml min^{-1} kg^{-1})	889 ± 622[a]	288 ± 267
Cmax (ng ml^{-1})	36 ± 7.0[a]	678 ± 762
Tmax (min)	30 ± 12	17 ± 5.0
$t_{1/2}$ (h)	1.7± 0.9[a]	2.4± 0.8

[a] $P < .05$ without metoprolol *vs* with metoprolol

[b] $P < .05$ S *vs* R

(n=6)

Table 5-2. Pharmacokinetic parameters of verapamil enantiomers after i.v. administration of racemic verapamil with and without metoprolol in rats. Data are expressed as mean ± SD.

	Without Metoprolol	With Metoprolol
S-Verapamil		
$AUC_{0-\infty}$ (μg h l^{-1})	309 ± 66[a b]	509 ± 165[b]
Cl_s (ml min^{-1} kg^{-1})	28 ± 4.6[a b]	18 ± 6.3[b]
$t_{1/2}$ (h)	1.5 ± 0.9[a]	2.4 ± 1.1
Vd_β (l kg^{-1})	3.4 ± 1.8[b]	3.9 ± 2.4[b]
R-Verapamil		
$AUC_{0-\infty}$ (μg h l^{-1})	212 ± 55[a]	293 ±78
Cl_s (ml min^{-1} kg^{-1})	41 ± 8.9[a]	31 ± 12
$t_{1/2}$ (h)	1.6 ± 0.9[a]	2.4 ± 1.0
Vd_β (l kg^{-1})	5.3 ± 2.5	5.9 ± 2.0

[a] $P < .05$ without metoprolol *vs* with metoprolol;

[b] $P < .05$ S *vs* R

(n=6)

Table 5-3. Protein binding of verapamil enantiomers, with and without metoprolol.

	Protein Binding, (% Unbound)			
Subject	S-V (without MET)	S-V (with MET)	R-V (without MET)	R-V (with MET)
1	10.2	12.4	7.6	7.1
2	12.8	11.9	8.1	8.6
3	12.7	13.2	10.4	9.2
4	12.5	13.6	10.7	9.1
Mean	12.1	12.8	9.2	8.5
SD	1.2	0.8	1.6	0.9

(V=verapamil, NV=norverapamil, MET=metoprolol)

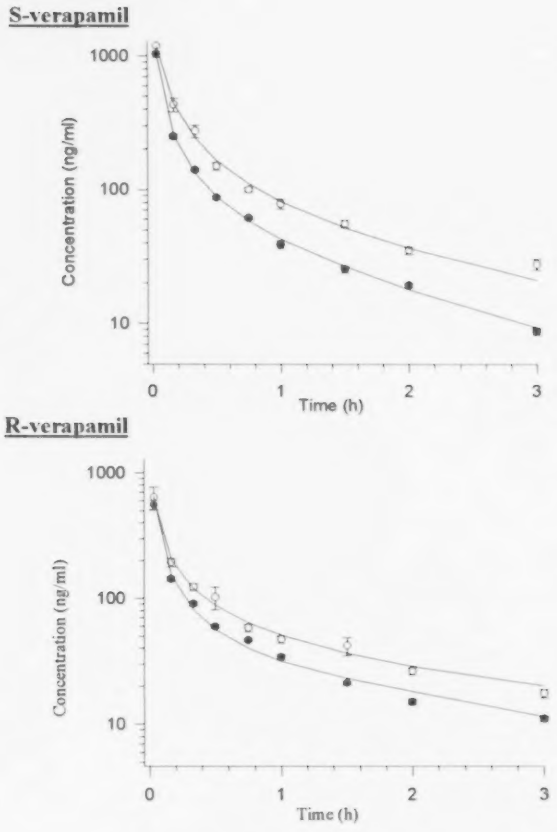

Figure 5-1. Plasma concentration *versus* time profile for enantiomers of verapamil after orals administration of racemic verapamil with and without metoprolol; With metoprolol = ○, Without metoprolol = ●. Each point is the group mean ± SEM. (n=6, no drug was detected at 5 h time point).

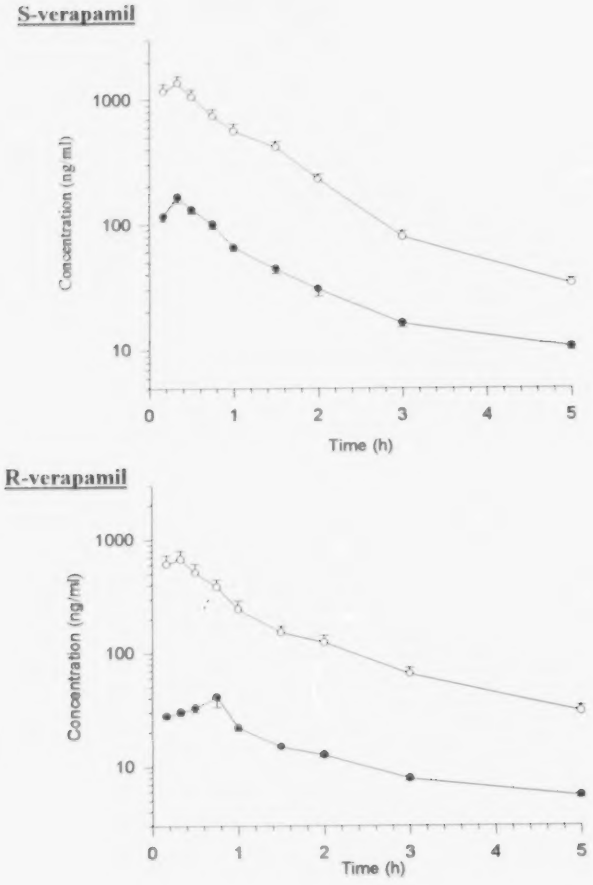

Figure 5-2. Plasma concentration *versus* time profile for enantiomers of verapamil after intravenous administration of racemic verapamil with and without metoprolol; With metoprolol = ○, Without metoprolol = ●. Each point is the group mean ± SEM. (n=6).

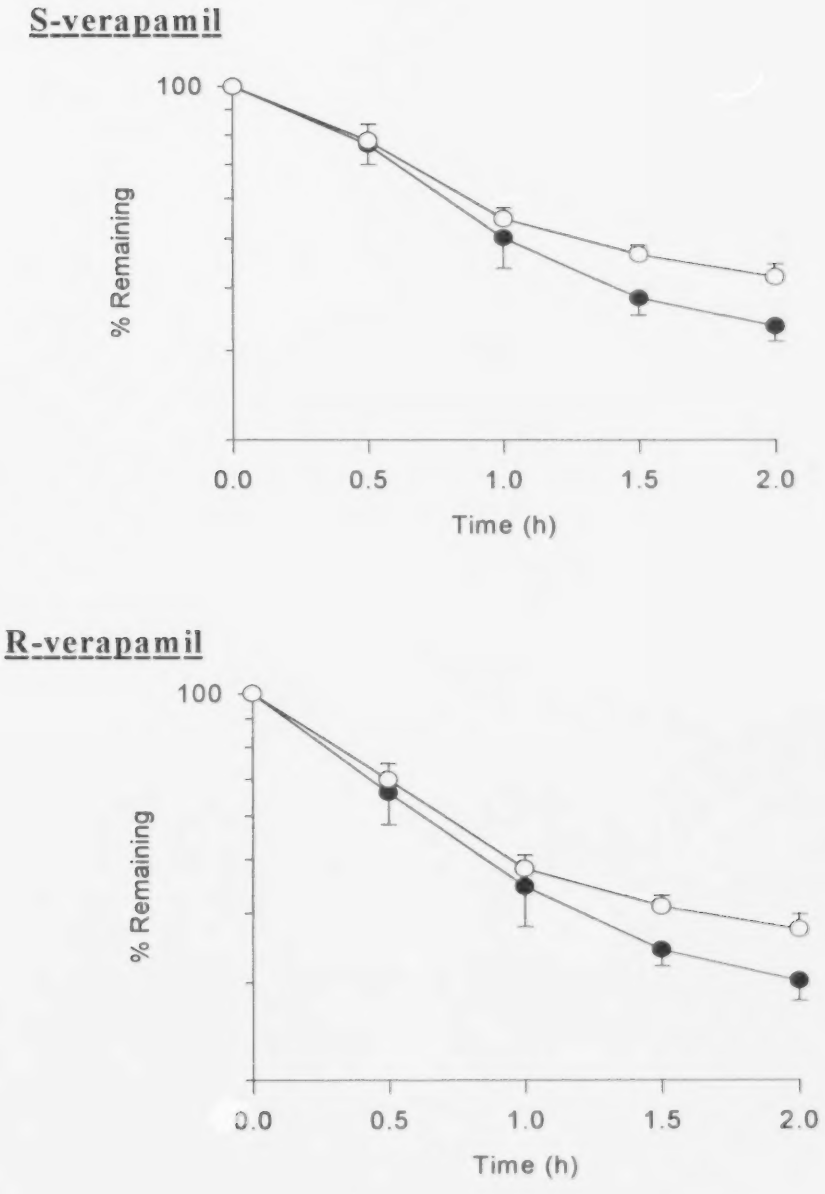

Figure 5-3. Percentage verapamil remaining *versus* time in microsomes with and without metoprolol: With metoprolol = ○, Without metoprolol = ●. Each point is the group mean ± SEM. (n=5).

SUMMARY AND CONCLUSIONS

Verapamil, a calcium channel blocking agent, is used in the treatment of angina pectoris, hypertension and supraventricular tachycardia. Although verapamil is manufactured and administered as the racemate, most of the calcium channel blocking activity resides with the S-enantiomer. Verapamil is almost completely absorbed when given orally but has about 20% bioavailibility because of extensive stereoselective first-pass metabolism. Thus, the conditions or drugs that can alter hepatic blood flow and/or enzyme activities have the potential to change the disposition of verapamil enantiomers. The purpose of this study was to examine the effects of some of these factors on the stereoselctive disposition of verapamil.

A sensitive and stereospecific reversed-phase HPLC method for the analysis of verapamil and norverapamil enantiomers was reported, using a chiral derivatizing agent. The derivatization procedure was optimized by making use of a microwave-facilitated reaction. The assay proved to be valid for the determination of verapamil and norverapamil enantiomers in clinical samples. Later, another stereospecific assay was developed using a single chiral stationary phase. This assay was simpler than the previous one.

The effect of input rate was investigated in humans after the administration of immediate-release (IR) and controlled-release formulations. This study

determined that the disposition of verapamil and norverapamil enantiomers was stereoselective regardless of the formulation administered and that there was a trend towards decreased systemic availability of the more active S-verapamil. The possible reasons that others [1-2] detected and we were unable to detect statistically significant differences between the products may include the low sensitivity of the assay and very higher intersubject variability. The higher intersubject variability in the present study may be due to the presence of both male and females subjects, since recent studies have shown gender-related differences in the stereoselective disposition of verapamil [3].

The disposition of verapamil enantiomers was investigated in rats after oral and i.v. administration. The kinetics of verapamil enantiomers was not previously studied in rats. In this study, we found that the elimination characteristics of verapamil enantiomers were comparable to humans as it was extensively and stereoselectively metabolized by rat. After oral administration the systemic availabilities of both enantiomers were low. The stereoselectivity in the systemic and presystemic clearance was opposite in rat than in human. Even though investigators [4] found considerable similarities in the metabolic pathways of the two species *in vitro*, and concluded that a similar set of cytochrome P-450 isozymes may be responsible for this biotransformation. The possible explanation of different stereoselectivities observed by the two species may be due to the structural differences in these enzymes and/or due to free fraction of the drug.

In the protein binding study, we observed a very high and stereoselective binding of verapamil in rat and human plasma. Consistent with the kinetics results, the stereoselectivity in the binding in rat plasma was opposite than in the human. We did not find any significant affect of age on the binding of verapamil enantiomers in both species. At the concentrations studied norverapamil and metoprolol did not effect the binding of verapamil.

From the results obtained in the metoprolol co-administration study in rats, it appeared that metoprolol has an effect on the disposition of verapamil enantiomers. In the i.v. experiment, it was found that metoprolol significantly decreased the Cl_s of verapamil enantiomers. The possible explanation of this may be the reduction in the hepatic blood flow after the administration of metoprolol. Since verapamil enantiomers have high extraction ratio, their Cl_s is predominantly dependent on the hepatic blood flow rate. Changes in their Cl_s, therefore, may be a reflection of changes in the hepatic blood flow. This decrease in Cl_s resulted in lengthening of the terminal $t_{1/2}$ of verapamil enantiomers without changing their volume of distribution. This was expected for a highly extracted drug like verapamil whose $t_{1/2}$ is dependent upon hepatic blood flow. Metoprolol also increased approximately 5 to 7 folds the average systemic availability of S- and R-verapamil. The possible explanation of this increase in the systemic availability of verapamil enantiomers may be the decrease in the intrinsic clearance. This was evident by the observed decreased Cl_o of verapamil enantiomers. To further investigate this effect, we studied the effect of metoprolol on the *in vitro*

metabolism of verapamil enantiomers in the rat liver microsomal preparation. Even though the loss of verapamil was generally slower in treatment as compared to controls, statistically significant differences were only found at the two last data points. This was probably due to: 1) the interaction was studied only in one concentration of metoprolol (2 mg/l) and there might be more pronounced differences if the study could have done at higher concentrations; 2) the results of the microsomal study may or may not reflect those of the intact animals.

In summary, the new findings reported as a result of this study include: 1) pharmacokinetics of verapamil and norverapamil is stereoselective in humans after the administration of IR and CR formulations and there is a trend of decreased systemic availability of the more active S-verapamil after CR administration; 2) the disposition of verapamil is stereoselective in Sprague-Dawley rats; 3) the protein binding of verapamil is stereoselective in humans and rat; age did not affect the binding in either species; 4) Sprague-Dawley rat can be use to study the pharmacokinetics of verapamil enantiomers and 5) Co-administration of racemic metoprolol significantly decreased the systemic and oral clearances of verapamil enantiomers in Sprague-Dawley rats.

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